

Cellular and serological responses to vaccination in chronic kidney
disease

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A thesis submitted for the degree of Master of Philosophy of The
Australian National University

January 2018

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Word count : 7592 words; excluding title pages, acknowledgements, table of contents, tables, figures, figure legends, and references.

E. N. da Silva

January 2018

Acknowledgements

I would like to thank my supervisor Professor Matthew Cook; Dr Krishna Karpe in the department of Renal Medicine at the Canberra Hospital who collaborated enthusiastically in the development and execution of the project; members of my supervisory panel, Professor Carola Vinuesa and Professor Chris Goodnow; Dr Jalila Alshekaili who provided invaluable advice regarding sample collection, assay development and analysis; Dr Alan Baker who assisted in recruiting participants for the 2012 influenza cohort and sample collection; Joyce Ho-Chinn and staff at the Occupational Medicine Unit at The Canberra Hospital who assisted with recruitment of healthy control participants; Dr Harpreet Vohra and Dr Michael Devoy, MCRF, John Curtin School of Medical Research, Australian National University, for assistance with flow cytometry acquisition; Dr MaryAnn Kuhl who kindly assisted in recruitment of healthy controls; Jennifer Stapleton and staff in flow cytometry at ACT Pathology; Candice Li and staff in immunoassay at ACT Pathology; Michael de Souza and staff in infectious serology at ACT Pathology; the staff in the Cook Lab, particularly Rochna Chand who performed the flow cytometry acquisition for the 2015-2016 cohort, as well as Wesley Lam, Kristy Lee, Zuopeng Wu, and Umang Sritsava who assisted with specimen processing and storage; Anastasia Wilson and Ann-Marie Hatch who assisted with recruitment and follow up; nurses Elise Field and Renee Choe in the Department of Immunology at the Canberra Hospital who assisted with hepatitis B vaccination; Megan Hughes, Zivai Mumbarese, Helen McFarlane in the Department of Renal Medicine at the Canberra Hospital for all of their work in patient recruitment, data collection and collaboration; Joyce Thanabal and all of the staff at the Canberra Community Dialysis Centre in Garran for their wonderful assistance in recruitment, sample collection, and data collection; Dr Katrina Randall and Dr Carolyn Hawkins for their mentorship and support; the organizing committee of the PathUpdate 2015 annual conference for providing me with an opportunity to present an earlier version of this work to a national collegiate audience; and, in particular, all of the volunteers who participated.

Abstract

Chronic kidney disease (CKD) and haemodialysis (HD) are associated with an increased risk of hepatitis B infection due to exposure to the virus during haemodialysis, in conjunction with impaired seroconversion to hepatitis B vaccination (HBV). Studies examining augmented vaccine schedules to enhance seroconversion have so far been inconclusive. Furthermore, the cellular defects responsible for impaired vaccine immunity observed in CKD and HD have not yet been identified. This project studied serological and cellular responses to HBV in CKD and HD to identify defects in vaccine-induced cellular responses that could account for impaired seroconversion and clarify the effects of an augmented vaccine dose schedule. These results were compared with responses to seasonal influenza vaccination. There was a clear benefit in rates and magnitude of seroconversion after an augmented 40mcg HBV dose schedule in CKD. This permitted comparison of responders and non-responders. Serological non-responders with CKD exhibited a reduction in CXCR3+CCR6- CXCR5+ memory T cells at baseline. Seasonal influenza vaccine elicited a plasmablast (PB) response in both healthy controls and CKD, but HBV elicited a poor plasmablast response in both groups. Both vaccinations induced activation of the CXCR3+CCR6- subset of circulating T follicular helper cells (cTFH) in healthy controls, and but this response was impaired in CKD after HBV, even with the augmented 40mcg HBV dose schedule, and appears not to be reversed by haemodialysis. Despite this, some patients receiving haemodialysis generate detectable post-vaccination HBsAb, and this correlates with activation within the CXCR3+CCR6- cTFH compartment. We concluded that cellular responses to seasonal influenza vaccine are preserved in CKD and haemodialysis. However, CKD confers a specific defect in cTFH activation that contributes to the impaired seroconversion to HBV, and this defect persists despite renal replacement therapy with haemodialysis.

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ABBREVIATIONS

CCR6	Chemokine, CC motif, Receptor 6
CCR7	Chemokine, CC motif, Receptor &
CKD	Chronic Kidney Disease
CXCR3	Chemokine, CXCR motif, Receptor 3
CXCR5	Chemokine, CXC motif, Receptor 5
cTFH	circulating T follicular helper cells
GC	Germinal centres
HBsAb	Hepatitis B surface antibody
HBV	Hepatitis B vaccine
HC	Healthy Controls
HD	Haemodialysis
ICOS	Inducible co-stimulator
IL-2	Interleukin 2
IL-6	Interleukin 6
IL-10	Interleukin 10
µg	micrograms
PB	Plasmablast(s)
PD-1	Programmed death-1
TFH	T follicular helper cells
TGF-β	Transforming growth factor - beta
TNFα	Tumour Necrosis Factor alpha
Treg	Regulatory T cells

Chapter 1: Literature Review

1.1 Introduction

End-stage renal disease confers a state of chronic immune deficiency, which results in poor response to standard vaccination regimens, and increased risk of infection, including chronic viral infections that can increase risk of cancer.¹ The risk of hepatitis B virus infection is particularly significant due to increased exposure to the virus during haemodialysis, and an increased risk of chronic infection and mortality.² Current guidelines recommend routine vaccination of dialysis patients with three or four “double” doses of HBV, equivalent to 40µg of HBV, however there is no universal dosing recommendation for pre-dialysis CKD patients.²⁻⁴ The Centers for Disease Control and Prevention recommends 20µg of HBV intramuscularly at 0, 1, 6 months in pre-dialysis patients. A significant proportion of CKD patients vaccinated prior to the commencement of dialysis remain at risk of contracting hepatitis B virus due to impaired seroconversion and rapid decline of protective titres.⁵ Several studies have sought to address the possible benefit of augmenting the pre-dialysis schedule to four 40µg doses, however the data are so far inconclusive.⁵⁻⁸ At a cellular level, the reasons for impaired serological response to HBV in CKD are poorly understood.

The benefit of an augmented HBV schedule in patients already receiving dialysis has been established by several studies^{5,6,9,10} and has been integrated into clinical practice guidelines.^{2,11} However, the cellular mechanisms underlying this benefit have not been identified. The performance of the seasonal influenza vaccine, in terms of seroconversion and protective antibody, appears to be adequate in

chronic kidney disease including in the context of haemodialysis,¹²⁻¹⁴ however the reason(s) for this, at a cellular level, have not been clarified.

1.2 The immune defects in chronic kidney disease

Various cellular defects in immunity have been identified in patients with CKD, including quantitative and functional defects in lymphocyte compartments¹⁵⁻¹⁸ secondary to increased activation and apoptosis.^{15,16,19} B cell compartment defects include reduced absolute numbers of total, innate, naïve and memory B cells.¹⁹ One possible explanation is reduced expression of B-cell activating factor of the TNF-family (BAFF)-receptor.¹⁰ T cell compartment defects include low absolute counts of total,¹⁵ naïve and central memory T cells of both CD4+ and CD8+ subsets.^{15,18} Some of these defects have been partly attributed to the metabolic derangements of uremia and hyperphosphatemia.¹⁸ At the same time, there is an inverse relation between glomerular filtration rate and markers of inflammation, with observed increased plasma levels of TNF α , IL-10, IL-12, IL-15, IL-8, IL-1 α and eotaxin in all CKD patients irrespective of progression to dialysis.²⁰⁻²² Possible explanations for these abnormalities include decreased cytokine clearance, increased gut permeability, periodontitis, and oxidative stress of uremia.²³⁻²⁵

1.3 The effects of haemodialysis on the immune system

Multiple immune defects have been demonstrated in patients with CKD who are receiving renal replacement therapy with haemodialysis, although it is not clear which of these defects are secondary to kidney disease or the process of haemodialysis. Haemodialysis results in increased serum levels of IL-1, in a cumulative fashion with repeated treatments, and exacerbates already elevated levels of TNF α seen in CKD²¹, indicating dialysis induces an acute inflammatory

state. *In vitro*, B cells from patients receiving haemodialysis display reduced T-dependent proliferation, impaired immunoglobulin production, and increased susceptibility to apoptosis²⁶. Both CD4+ and CD8+ circulating T cells are reduced in number in patients receiving haemodialysis, but particularly CD4+CD45RA+ naïve T cells²⁷. There is impaired T cell proliferation in response to staphylococcus A *in vitro*²². CD4+ T cells demonstrate reduced key surface expression markers including the co-stimulatory molecule CD28, and markers of activation CD25 and CD69²⁸, when compared to cells from both healthy controls and CKD patients pre-dialysis. Interestingly, these defects are partially ameliorated both *in vivo* and *in vitro* by treatment with recombinant erythropoietin, suggesting these defects may derive from impaired kidney function rather than the process of hemodialysis^{26,27,29}.

1.3.1 Vaccine responses in patients on haemodialysis

Patients receiving haemodialysis have a lower rate and magnitude of seroresponse to hepatitis B vaccine compared to healthy controls.^{6,8} Non-responders to hepatitis B vaccine demonstrate higher levels of inducible IL-6 and TNF α *in vitro*, whereas responders demonstrate higher levels of inducible IL-10³⁰. Data regarding the serological responses to trivalent seasonal influenza vaccine in haemodialysis patients are conflicting^{12,14,31,32}, but point towards adequate seroprotection despite lower titres of vaccine-specific antibody, and suggest that other factors, such as older age, may contribute to poor vaccine response in this patient group³¹.

Patients on haemodialysis have impaired vaccine responses to other vaccines including pneumococcal 23-valent polysaccharide vaccination³³, when compared

to both healthy controls and patients with CKD who have not yet progressed to dialysis, with more rapid decline of titre with time. While haemodialysis patients have good initial responses to tetanus toxoid vaccine, there is a more rapid decline seen in vaccine specific antibody titres over time³⁴. Initial response to tetanus vaccine does not correlate with response to hepatitis B vaccination in this patient group³⁴.

1.4 Vaccine induced antibody responses

High-affinity antibody responses depend on interactions between T and B cells in specialized microanatomical sites called germinal centers (GC), located in secondary lymphoid organs.^{35,36} T follicular helper cells (TFH) are a subset of CD4 T cells characterized by high level expression of CXCR5, ICOS and PD-1, and the capacity to secrete IL-21.³⁷ TFH provide help to B cells at priming and to centrocytes in GC that results in their differentiation into memory B cells and long-lived plasma cells. Memory B cells are thought to serve as precursors of long-lived plasma cells after re-encounter with antigen. Recently, progress has been made in monitoring these cellular events in peripheral blood. 5-8 days after booster parenteral vaccination PB can be detected in the peripheral circulation.³⁸⁻⁴⁰ These PB are antigen-specific, predominantly of IgG isotype, and their peak in peripheral blood correlates with serological response.^{38,39,41} Similarly, a peripheral cellular signature of events within GC has been pursued by enumerating putative circulating counterparts of TFH.⁴²⁻⁴⁵ These cells are memory CD4⁺ T cells expressing the B-cell-zone homing chemokine receptor (CXCR)-5,⁴⁶ high levels of Inducible Co-stimulator (ICOS)⁴² which is critical for TFH development,⁴⁷ Programmed Cell Death (PD)-1,⁴⁸ and a marker of recent T cell activation via the T cell receptor.

Chemokine receptors CXCR3 and CCR6 have previously been identified as surrogate markers of effector T cell differentiation including within GC (CXCR3⁺CCR6⁻ (Th1), CXCR3⁻CCR6⁻ (Th2) and CXCR3⁻CCR6⁺(Th17)),^{49,50} and have also been demonstrated to distinguish functional cTFH subsets.⁴³ cTFH that express CXCR3 but not CCR6 transiently increase 7 days following influenza vaccination, are antigen-specific, upregulate expression of ICOS and PD-1, and correlate with both serological response and the day 7 peak in PB.⁴² There are no previous studies examining dynamic PB or TFH responses to vaccination in CKD or HD patients, nor after HBV.

Regulatory T cells (Tregs) are FOXP3⁺ CD4⁺ T cells providing immune homeostasis, via modulation of adaptive immune responses through a variety of mechanisms including the production of inhibitory cytokines like IL-10 and TGF- β , and depletion of IL-2 in the local milieu via high surface expression of the IL-2 high affinity receptor, CD25⁵¹. Tregs can be broadly characterized as either thymic or inducible Tregs, with the latter forming in secondary lymphoid organs⁵². Murine studies suggest that Tregs interfere with vaccine responses, which can be ameliorated by depletion of Tregs by various strategies⁵¹. Furthermore, a recent study demonstrated a negative correlation between baseline Tregs in the peripheral circulation and measles specific antibody titre taken 4 weeks after measles vaccine in infants⁵³. Induction of peripheral Tregs has not been studied following HBV or seasonal influenza vaccination, but could potentially be a source of impaired vaccine responses, including to HBV in CKD and HD.

Chapter 2: Methods

2.1 Patients

The study was approved by The Canberra Hospital Human Research and Ethics Committee. All recruits provided written informed consent to participate.

Between 2011 and 2016 patients with stage 3 (30-59 ml/min/1.73m²) or 4 (15-29 ml/min/1.73m²) CKD requiring HBV or seasonal influenza vaccine for routine care were recruited through the Department of Renal Medicine at The Canberra Hospital. Exclusion criteria for CKD patients were dialysis or kidney transplant. HC requiring routine HBV or seasonal influenza vaccine for occupational reasons were recruited via the Occupational Medicine Unit at the Canberra Hospital, from the author's colleagues, and via a private geriatrics outpatient clinic at National Capital Private Hospital, Canberra. Patients and HC recruited to receive the seasonal influenza vaccine were vaccinated in either 2012 or 2013 only. All other patients and HC recruited during this period received HBV.

In 2015 and 2016 patients with CKD receiving haemodialysis were also recruited to receive either booster HBV or seasonal influenza vaccine, for routine clinical care. Exclusion criteria for all groups included cancer, infection, other chronic inflammatory disease, immunosuppressive medication or other defined immunodeficiency.

2.2 Vaccines

Undetectable HBsAb titre (<10mIU/ml, ARCHITECT anti-HBs, Abbott, Dublin, Ireland) was a pre-requisite to enrolment into the HBV arm of the study. HC received standard schedule HBV (recombinant hepatitis B surface antigen, Engerix-B, GlaxoSmithKline, Brentford, United Kingdom) consisting of three doses

of 20 μ g, at 0, 1 and 4 months or at 0, 1 and 6 months. CKD patients receiving HBV were randomized to receive either four doses of 20 μ g or four doses of 40 μ g of HBV, at 0,1,2 and 6 months. Dialysis patients receiving HBV were administered a single booster dose of 40 μ g.

Trivalent, split virion, inactivated influenza vaccine was used in the study as follows: 2012 - Fluvax® (CSL Behring, Melbourne, Australia) - A/California/7/2009, A/Perth/16/2009, B/Brisbane/60/2008); 2013 - Fluvax® (CSL Behring) - A/California/7/2009, A/Victoria/361/2011 and B/Wisconsin/1/2010; 2015 - Influvac® (BGP Products, Mylan, Zurich, Switzerland) - A/California/7/2009, A/Switzerland/9715293/2013, B/Phuket/3073/2013 ; and 2016 - Influvac® (BGP Products) - A/California/7/2009, A/Hong Kong/4801/2014, B/Brisbane/60/2008.

2.3 Serological analysis

CKD patients and healthy controls who received the full HBV schedule underwent serum collection four weeks after the final dose for HBsAb titre. Haemodialysis patients who received an HBV booster also underwent serum collection four weeks later for HBsAb titre. Seroconversion was defined as a titre \geq 10 mIU/ml. Serum collection was repeated in CKD patients and HC between three and five years later to determine longitudinal HBsAb titre.

2.4 Sample collection for cellular analysis

Peripheral blood was collected before (median 0 days, range -29-0 days, mean 1.5 days) and 7 days after (median 7, range 6-13, mean 7.5 days) vaccination, for all enrolled participants after either seasonal influenza vaccine or the first dose of HBV. Samples were ficoll separated (Ficoll-Paque PLUS, GE Healthcare, Chicago, IL, USA), and isolated PBMCs were washed and then frozen in DMSO at -80°C for future use.

2.5 Lymphocyte analysis with flow cytometry

Samples were thawed and analyzed using FACS for PB, cTFH and Tregs in batches, ensuring paired samples (baseline and day 7) for any given patient were analyzed in the same batch. Antibody panels used for staining cells in preparation for flow cytometry are outlined in Table 1. For the analysis of circulating plasmablasts and memory B cells, samples were labelled using anti-CD3 PerCP (12.5µg/ml), CD14 PerCP (25µg/ml), CD19 PE-Cy7 (25µg/ml), CD38 V450 (25µg/ml), CD27 APC (6µg/ml), HLA-DR APC-H7 (50µg/ml), followed by fixation and permeabilization (Cytofix/Cytoperm) (all BD biosciences, Franklin Lakes, NJ, USA), then labelled for intracellular immunoglobulin expression using anti-IgG PE (commercial concentration not available (NA)), IgA biotin (500µg/ml) (both BD biosciences) and IgM FITC Dako, Denmark (NA). Cells were then stained with streptavidin-conjugated V500 in permeabilization-compatible wash, before suspension in phosphate buffered saline (PBS) with 2% fetal bovine serum (FBS)(GIBCO/LifeTechnologies, Waltham MA, USA). In a separate experiment, samples collected between 2011 and 2013 were stained for cTFH using anti-CD3 FITC (100µg/ml), CCR6 PE (200µg/ml), CCR7 PE-Cy7 (NA) (all from BD biosciences), CD4 APC-Cy7 (NA), CD45RA Pacific Blue (500µg/ml), CXCR5

PerCP/Cy5.5 (NA), CXCR3 Brilliant Violet 510 (NA) (all from Biolegend, San Diego, CA, USA), PD-1 biotin (500µg/ml) and streptavidin-APC (200µg/ml) (eBioscience, Thermo Fisher Scientific, Waltham, MA, USA). In another experiment, samples collected in 2015 and 2016 were stained for cTFH using anti-CD3 AF700 (500µg/ml), CXCR5 PerCP-Cy5.5 (both from Biolegend), CD45RA FITC (NA) (Miltenyi Biotec, Glerdisch Gladbach, Germany), PD-1 Brilliant Violet 421 (NA)(BD biosciences), ICOS biotin (500µg/ml) (eBioscience), streptavidin-APC, CD4 APC-Cy7, CXCR3 Brilliant Violet 510, CCR6 PE. In another experiment, samples collected from 2015 and 2016 were stained for Tregs using anti-CD3 AF700, CD4 APC-Cy7, CD45RA FITC, CXCR5 PerCP-Cy55, CD25 APC (12µg/ml)(BD biosciences), CD127 Brilliant Violet 510 (NA)(BD biosciences), FoxP3 PE (NA)(BD biosciences), PD-1 Brilliant Violet 421, ICOS biotin, and streptavidin-PE-Cy7 (BD biosciences). After staining, samples were washed in (PBS) with 2%(FBS), and fixed (Cytofix, BD biosciences). Samples were re-suspended in PBS with 2% FBS for acquisition. All samples were acquired using a FACSCanto II or Fortessa (BD Biosciences). FACS data were analyzed using FlowJo (Version 10, Treestar Inc, Ashland, OR, USA). After gating broadly on viable leukocytes on forward and side scatter, and negative gating for CD3⁺ and CD14⁺ cells, PB were identified as CD19^{lo-hi} CD38^{hi} CD27^{hi} HLA-DR⁺, and isotyped according to cytoplasmic expression of IgG, IgA and IgM. cTFH cells were identified gating on lymphocytes on forward and side scatter parameters, followed by gating on CD3⁺ CD4⁺ CD45RA⁻ CXCR5⁺ cells, and, for the 2011-2013 cohort, analyzed for CXCR3, CCR6, PD-1 and CCR7 expression, and PD-1 mean fluorescence intensity. For the 2015-2016 cohort, cTFH were analyzed for CXCR3, CCR6, PD-1, and ICOS expression, and both PD-1 and ICOS mean

fluorescence intensities. Samples from the 2015-2016 cohort were also analyzed for Tregs by gating on lymphocytes, CD3⁺, CD4⁺, CD127^{lo}, CD25^{hi}, FOXP3⁺ cells, and analyzed for expression of ICOS and PD-1.

Randomly selected samples underwent repeat analyses using either panel, to determine assay reproducibility and validity. To confirm the validity of the assay on frozen and thawed samples, paired analyses were performed on fresh and frozen samples from a single donor.

Table 1. Antibody panels and fluorochromes used for flow cytometry experiments.

PLASMABLAST STAINING PANEL	
CD19	PE-Cy7
CD27	APC
CD38	V450
HLA DR	APC-H7
CD3	PerCP
CD14	PerCP
IgM	FITC
IgG	PE
IgA	Biotin
Streptavidin	V500

TFH STAINING PANEL 2011-2013	
ANTIBODY	FLUOROCHROME
CD3	FITC
CD4	APC-Cy7
CD45RA	PacBlue
CXCR5	PerCP/Cy5.5
CCR7	PE-Cy7
CCR6	PE
CXCR3	BV-510
PD-1	Biotin

TFH STAINING PANEL 2015-2016	
ANTIBODY	FLUOROCHROME
CD3	AF700
CD4	APC-Cy7
CD45RA	FITC
CXCR5	PerCP/Cy5.5
ICOS	Biotin
CCR6	PE
CXCR3	BV-510
PD-1	BV-421

Treg STAINING PANEL 2015-2016	
ANTIBODY	FLUOROCHROME
CD3	AF700
CD4	APC-Cy7
CD45RA	FITC
CXCR5	PerCP/Cy5.5
CD25	APC
CD127	BV510
FOXP3	PE
PD-1	BV-421

2.6 Lymphocyte assays

Routine blood samples were collected at baseline from CKD patients and controls enrolled to receive HBV between 2011 and 2013, for full blood count and lymphocyte subsets using dual platform analysis (Beckman Coulter LH750 Hematology Analyzer and FacsCantoII, BD Biosciences), and serum immunoglobulins (Architect, Abbott). Absolute lymphocyte counts were used in conjunction with experimental T cell panel analyses to calculate memory T cell parameters.

2.7 Statistical Methods

Statistical analyses were performed using Prism (Version 6; GraphPad Software Inc). p values < 0.05 considered significant.

Chapter 3: Results

3.1 CKD Cohort (2011-2016)

3.1.1 Enrolment

57 patients with stage 3 or 4 CKD were enrolled between 2011 and 2016 inclusive. Patient demographics and characteristics are shown in Table 2. 22 patients had diabetic nephropathy, of which 18 were taking insulin, 7 of whom were also on oral hypoglycaemic medication, and three were taking oral hypoglycemic medication only. Only one patient with diabetic nephropathy was not taking either medication. Other causes of renal disease were membranous glomerulonephritis (4), IgA nephropathy (4), renovascular disease (4), vasculitis (2), focal segmental glomerulosclerosis (2), mesangiocapillary glomerulonephritis (1), lupus nephritis (1), hypertension (1), polycystic kidney disease (1), embolic disease (1), obstructive uropathy (1), and unknown cause (13). Of these patients, two were taking insulin and oral hypoglycaemic medication (both with unknown cause of renal disease), and three were taking oral hypoglycaemic medication only (two patients with IgA nephropathy, and one with renovascular disease). Patient enrolment and sample collection are summarized in Figure 1.

Table 2: Patient demographics in the CKD cohort (2011-2013) according to vaccine groups. CKD – chronic kidney disease; HC – healthy controls; *Mann-Whitney t-test of medians.

	CKD	HC	p value*
hepatitis B vaccine			
patients (n)	57	12	
age (median, years)	69	53	0.1390
age (range, years)	34-85	21-93	
male:female	44:13	3:9	
seasonal influenza vaccine			
patients (n)	6	17	
age (median, years)	60.5	53	0.1615
age (range, years)	27-78	26-76	
male: female	5:1	9:8	

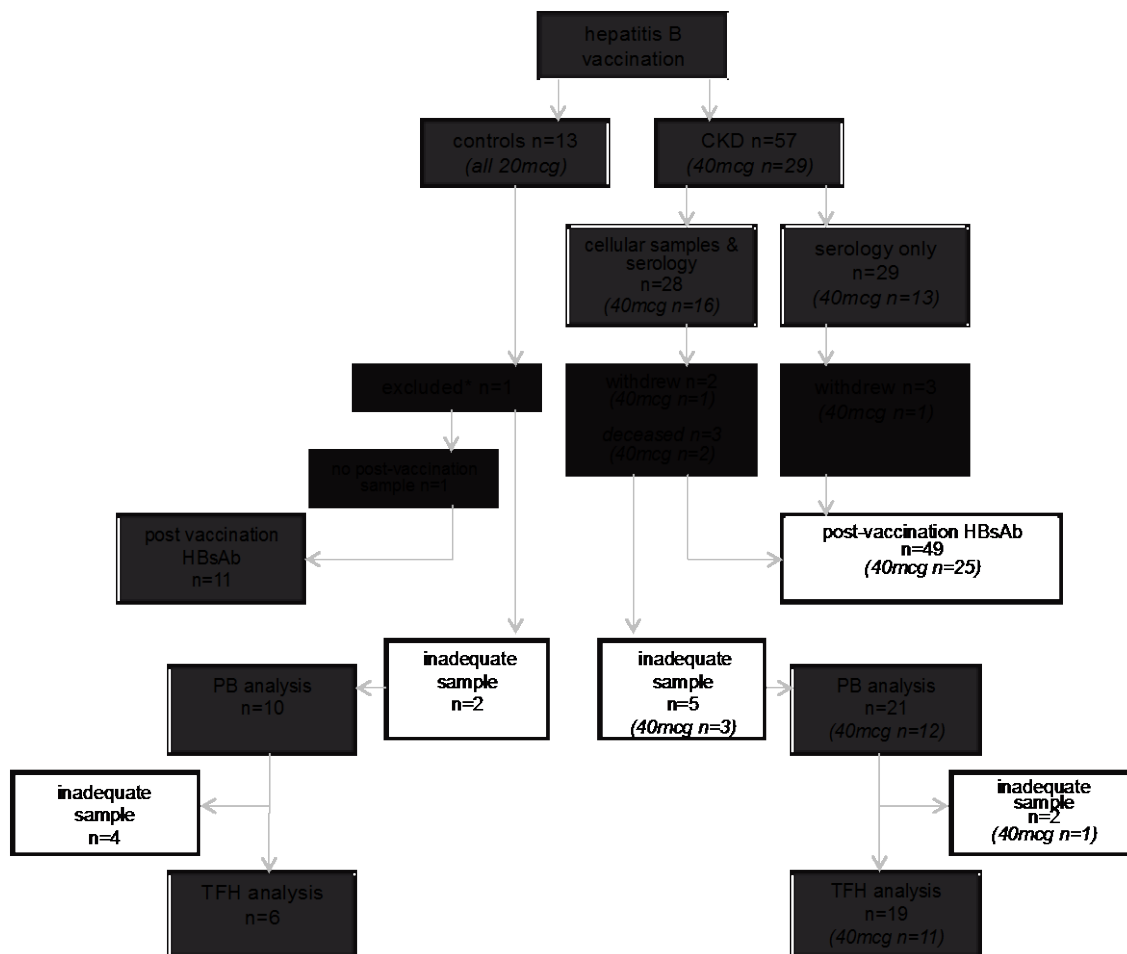


Figure 1. Summary of enrolment and exclusion

Flow diagram of enrolment and sample collection for healthy controls and CKD patients who received hepatitis B vaccine. All healthy controls received a standard dose of hepatitis B vaccine of 20µg, and were recruited for both cellular and serological sample collection.

*on Methotrexate; CKD - chronic kidney disease; HBsAb – hepatitis B surface antibody; PB – plasmablast; TFH – follicular helper T cell.

3.1.2 Lymphocyte subset analysis

Lymphocyte subset analysis was performed only for CKD patients and healthy controls enrolled between 2011 and 2013. At baseline, patients with CKD were lymphopenic relative to healthy controls (HC) (Fig. 2a). Total B cells were significantly reduced in CKD (Fig. 2b) due to reductions in memory and switched memory B cells. Median total B cell count in CKD ($0.08 \times 10^9/\text{L}$) was below the lower limit of the locally derived normal range ($0.14 - 0.54 \times 10^9/\text{L}$). CKD patients also had significantly lower absolute counts of total and CD4+ T cells (Fig. 2c). More detailed analysis of memory T cells revealed a trend towards a reduction in naïve CD4+ T cells (Fig. 2d), as reported previously.¹⁸ Both HC and CKD demonstrated median absolute CD8+ T cell counts below the lower limit of the diagnostic assay range ($0.3-0.9 \times 10^9/\text{L}$), consistent with a decline in this population with age (Fig. 2c). Central memory CD4+ T cells appeared significantly reduced in CKD, however absolute counts were low in both CKD and HC (Fig. 2d). Despite this finding, further analysis demonstrated a correlation between age and total T cells, and age and CD4+ T cells in healthy controls, but not between age and CD8+ T cells (Fig. 3). Age did not correlate with total T cells, CD4+ T cells or CD8+ T cells in CKD patients. There was no correlation between age and total B cells, or age and memory B cells, in either HC or CKD (*data not shown*). Median values for serum immunoglobulins (Ig) were within normal ranges in both HC (median values – IgG 8.83g/L, IgA 2.13g/L, IgM 0.88g/L) and CKD (median values - IgG 10.14g/L, IgA 2.59g/L, IgM 0.87g/L) (Fig. 4). There was no correlation between age and serum immunoglobulins in either HC or CKD (*data not shown*).

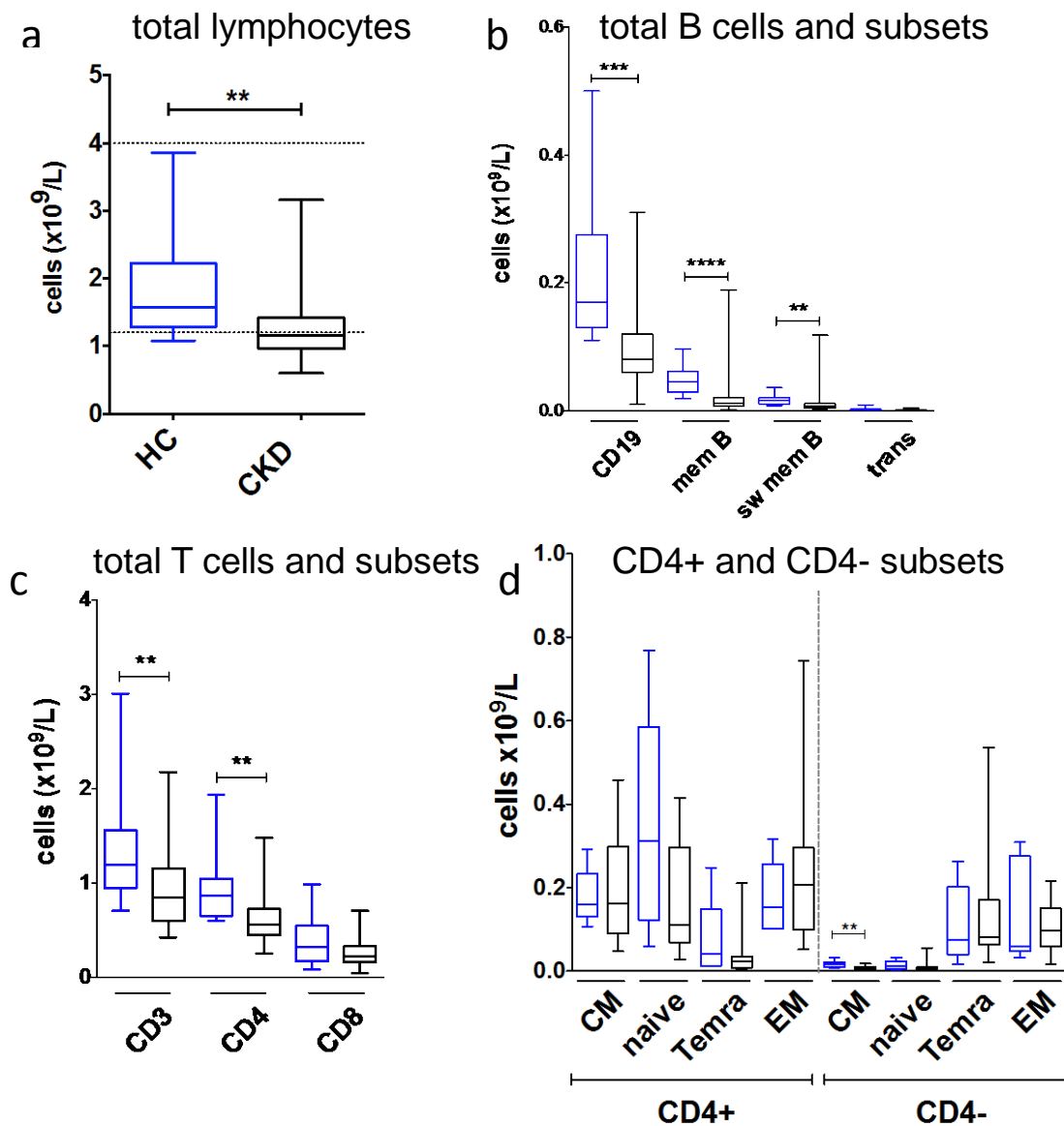


Figure 2. Baseline lymphocyte subsets

a-c. Peripheral blood lymphocyte analysis performed on baseline samples collected from healthy controls (HC; blue) and CKD patients (black) who received hepatitis B vaccination. Absolute counts (HC, $n=12$; CKD, $n=25$) for total lymphocytes (**a**), B cell subsets (**b**), and T cells subsets (**c**). **d.** Memory T cell subsets (HC, $n=6$; CKD, $n=16$). CM, central memory; EM, effector memory; mem B, memory B cells; sw mem B, switched memory B cells; trans, transitional B cells. All analyses performed using Mann-Whitney test of medians.

**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

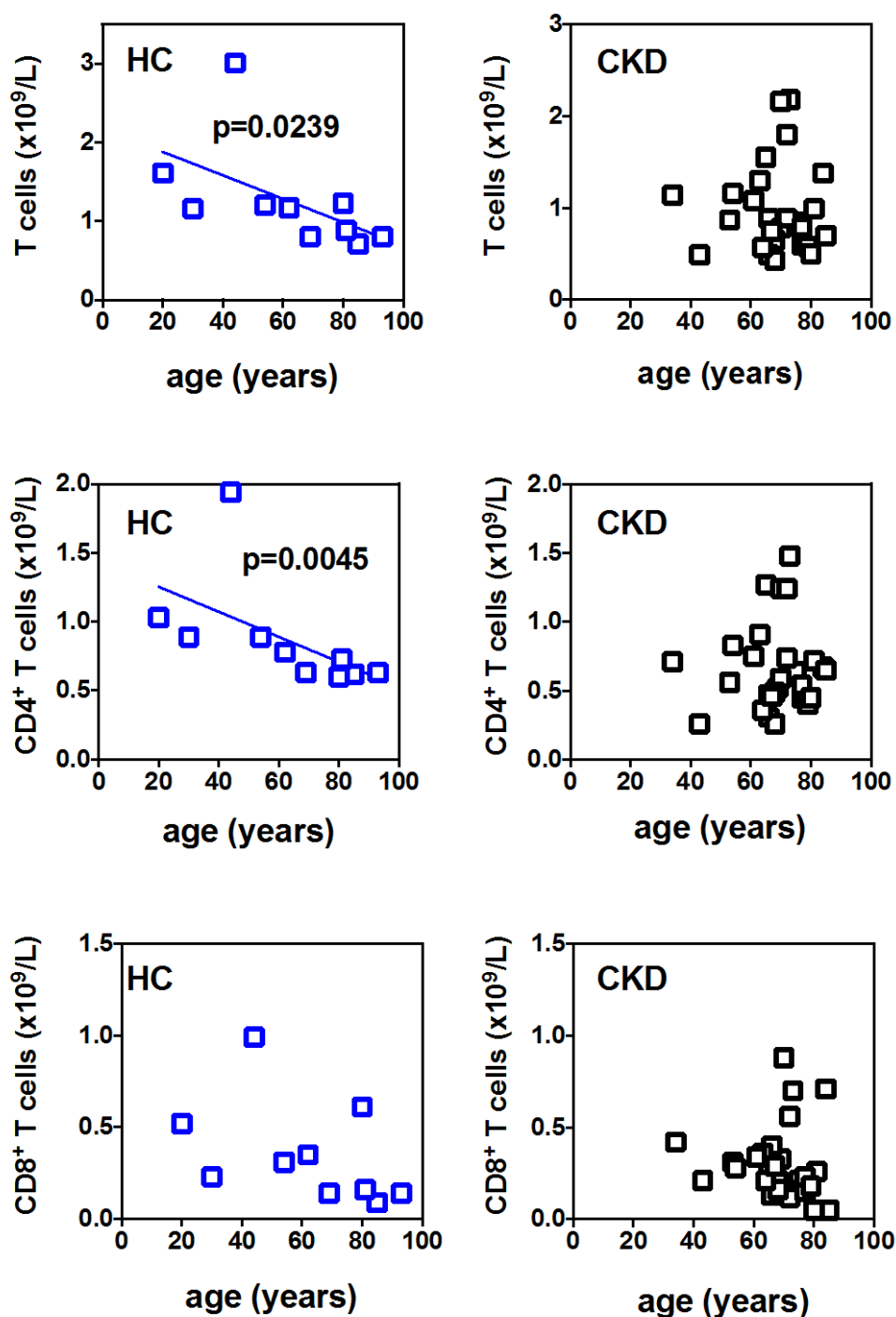


Figure 3. Age related changes in lymphocytes

Baseline total T cells, CD4⁺ T cells and CD8⁺ T cells, compared to age, in healthy controls and CKD patients who subsequently received hepatitis B vaccination. HC, healthy controls (blue squares, n=10), CKD, chronic kidney disease (black squares, n=27). Correlation analysis performed using Spearman test. Solid lines represent linear regression analysis.

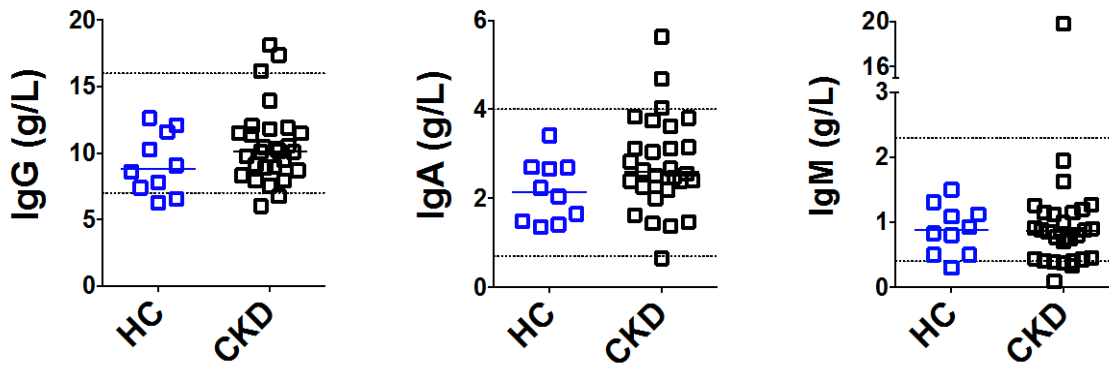


Figure 4. Baseline serum immunoglobulins.

Immunoglobulin (ig) measurements in healthy controls (HC, blue, n=10) and CKD patients (black, n=28) who subsequently received hepatitis B vaccination. Dashed horizontal lines represent working range of serological assay. Horizontal bars are medians, statistical analysis performed using Mann-Whitney test.

3.1.3 Post-vaccination hepatitis B serology

As noted in earlier studies, there was a significant incidence of serological non-responsiveness in CKD (Fig. 5a) (47% versus 27% in HC), although CKD responders generated titres of similar magnitude to HC. The median post-vaccination hepatitis B surface antibody (HBsAb) titre, including serological non-responders, was not significantly different between all CKD and HC. However, in CKD patients, the 40µg dose schedule resulted in a significantly higher median post-vaccination HBsAb titre (95mIU/ml vs <10mIU/ml), as well as reduced the rate of non-responsiveness (24% vs 71%) compared to the 20µg dose group). Furthermore, only two (8%) of the patients who received the 20µg schedule attained an HBsAb greater than 100mIU/ml, compared to 12 (28%) of those who received the 40µg schedule. Hence, overall, the 40µg schedule in CKD resulted in rates and magnitude of seroconversion comparable to HC. Thus, the serological defect observed in CKD with a 20µg dose schedule could be overcome by an augmented 40µg dose schedule.

Age influenced seroconversion independently of CKD. In HC, there was an inverse correlation between age and post-vaccination HBsAb titre (Fig. 5b), but this was not observed in CKD, even in the 40µg schedule group. Despite the negative effect of CKD on seroconversion, there was no direct relationship observed between eGFR and post-vaccination HBsAb titre (Fig. 6).

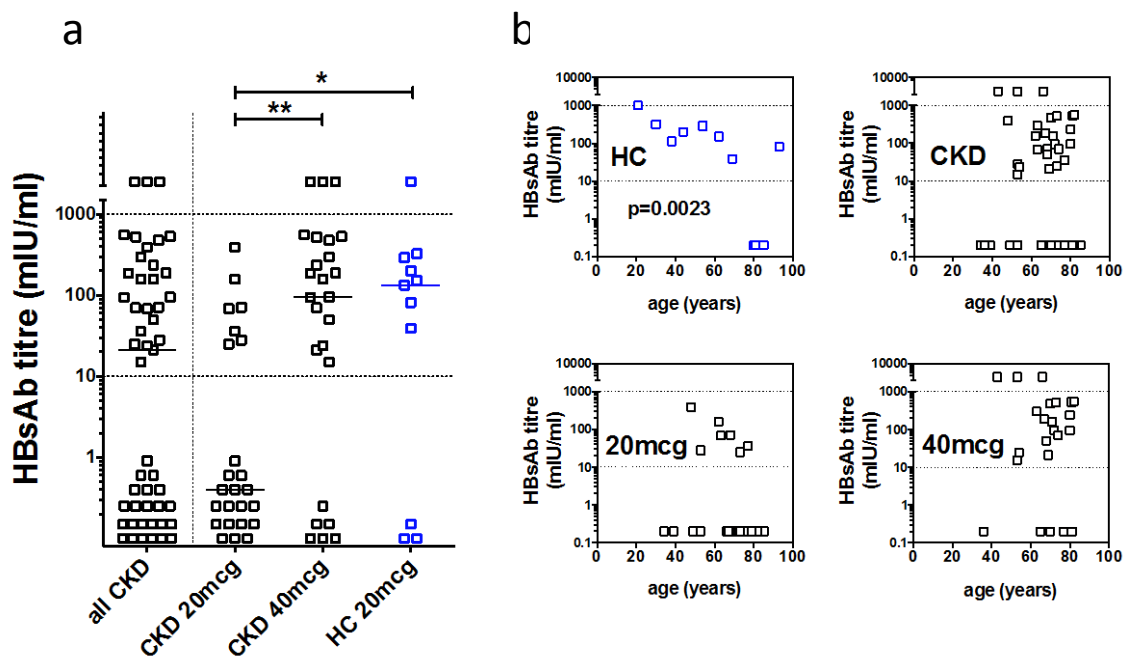


Figure 5. Post -vaccination HBsAb titers.

a. Post-vaccination HBsAb titer for CKD patients (black symbols; n=49) and healthy controls (HC; blue symbols, n=11) who completed primary hepatitis B vaccination schedule. Horizontal dotted line represents working range of serological assay. Horizontal bars indicate medians. Statistical analyses performed using Mann-Whitney test of medians, *p=0.0498, **p=0.0028. **b.** Correlation analysis of age and post vaccination HBsAb titer in healthy controls (n=11) and CKD patients (n=49), for both 20mcg CKD group (n=24) and 40mcg CKD group (n=25). p-values determined using Spearman test.

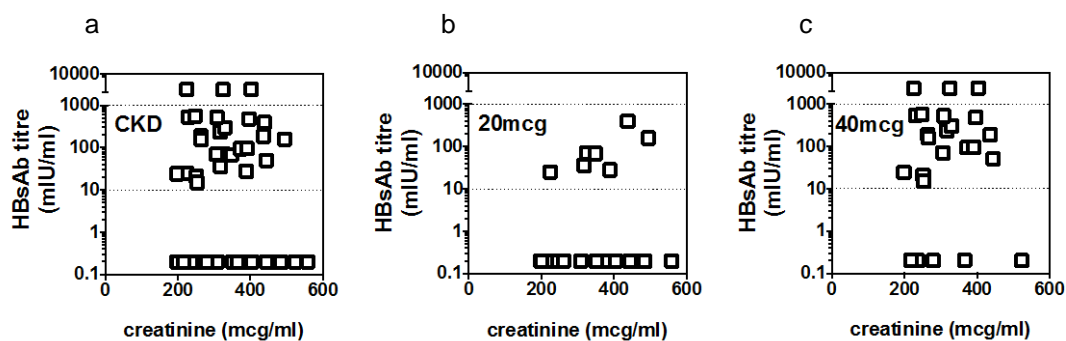


Figure 6. a-c. Post-vaccination HBsAb titer compared by eGFR in (a) all CKD patients, and CKD patients who received the (b) 20 μ g or (c) 40 μ g vaccine schedule. Horizontal dashed lines represent upper and lower limits of HBsAb assay detection. Statistical analyses performed using Spearman test. HBsAb, hepatitis B surface antibody; eGFR, estimated glomerular filtration rate.

3.1.4 Longitudinal hepatitis B serology

23 CKD patients and 10 healthy controls, who were recruited to provide both serological and cellular samples between 2011 and 2014, and had provided a 6 week post vaccination HBsAb titre, were contacted in 2017 to request longitudinal serology (HbsAb). 12 CKD patients and 5 healthy controls provided a repeat serum sample for longitudinal HBsAb titre. Of these, 7 CKD patients and 4 healthy controls were initial serological responders and were included in further analysis. Longitudinal serology for these initial responders is shown in Figure 7. While the numbers available for analysis were small, the decline in HBsAb titre was significant in CKD, but not HC. The time elapsed between serological measurements was slightly longer for CKD patients (average 1677 days, median 1648 days, range 1330-2150 days) compared to healthy controls (average 1459 days, median 1414 days, range 1052-1956 days).

Of the other CKD patients considered for longitudinal serology, 7 were deceased, 2 declined, and 2 transferred to other institutions. Of the other healthy controls, 4 were lost to follow up and 1 declined.

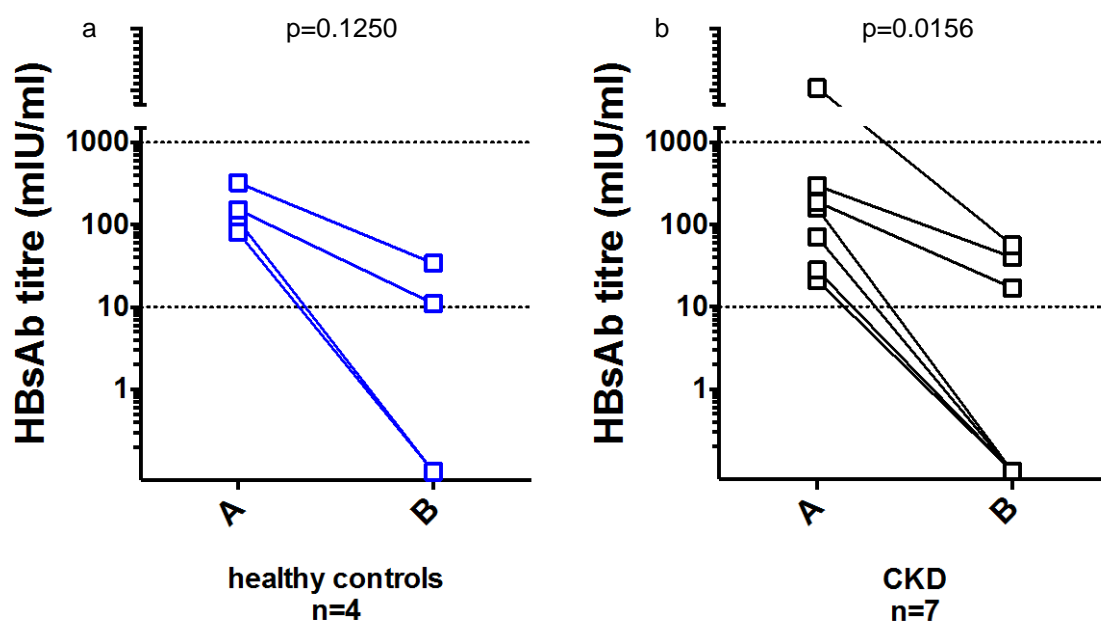


Figure 7. Longitudinal HBV serology for **a.** healthy controls and **b.** CKD patients, who initially responded to HBV. Wilcoxon signed rank test. Time between initial serology (A) taken six weeks after primary Hepatitis B vaccination course, and longitudinal serology (B) was longer for healthy controls (average 1677 days, median 1648 days) than CKD patients (average 1459 days, median 1414 days). HBsAb – hepatitis B surface antibody; CKD – chronic kidney disease.

3.1.5 Assay Validation

Paired analyses of fresh and frozen/thawed samples of peripheral blood indicated relative preservation of lymphocyte subsets, including rare events such as isotype-specific switched memory B cells and plasmablasts, and expression of PD-1 and ICOS on T cell subsets (*data not shown*). However, analysis of lymphocyte forward and side scatter characteristics demonstrated a second smaller lymphocyte population in frozen/thawed samples, which was not seen in fresh samples, consistent with dead or dying cells. Hence, subsequent lymphocyte gating excluded this population for all subsequent analyses (Fig. 8).

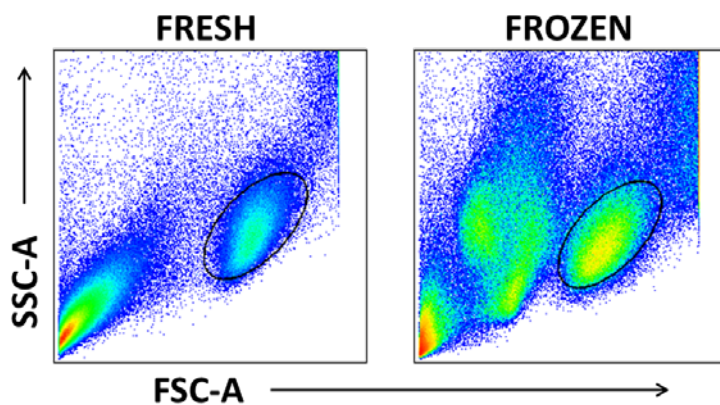


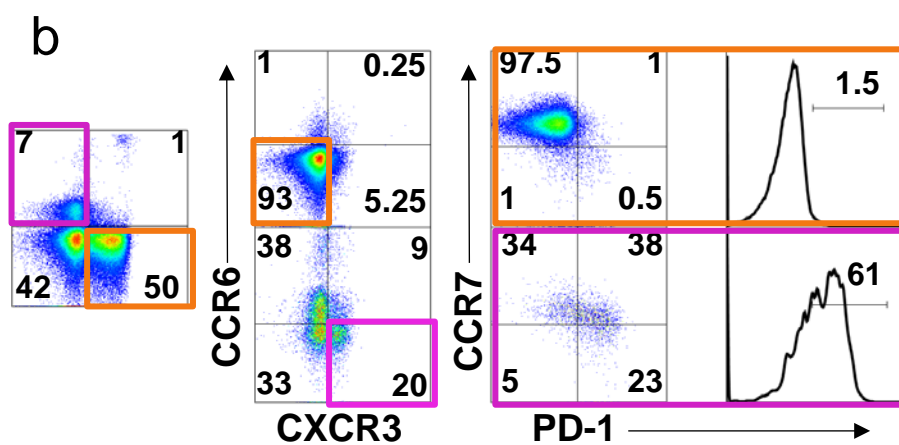
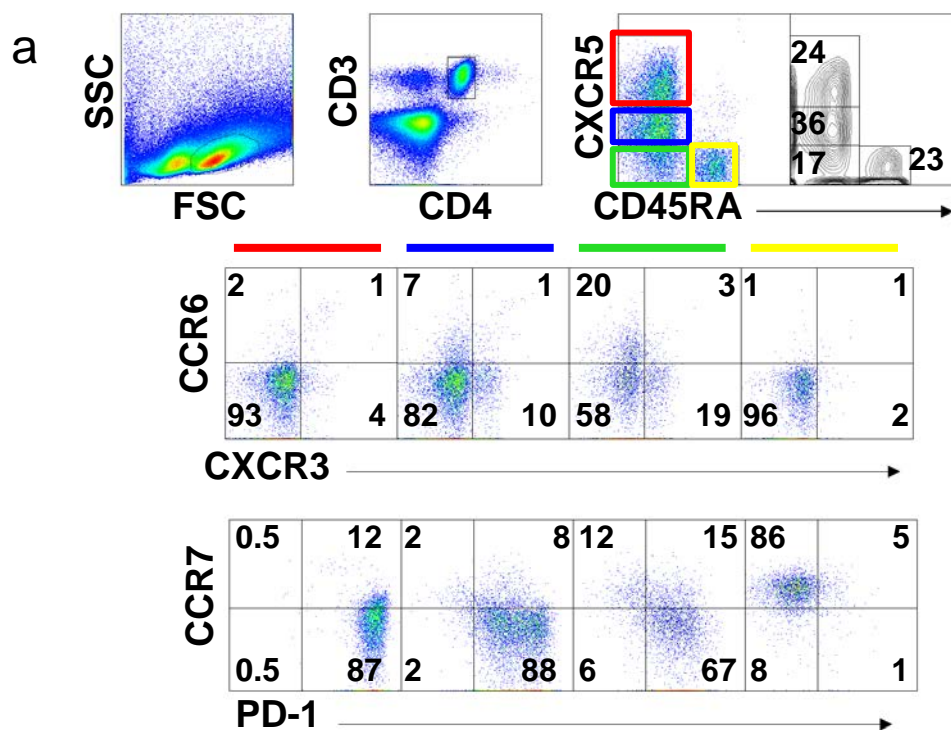
Figure 8. Paired analyses of fresh and frozen/thawed samples demonstrate an addition small lymphocyte population in the frozen/thawed sample (*right FACS plot*) which is not seen in the fresh sample (*left FACS plot*).

3.1.6 Baseline cTFH analysis

The study next examined whether CKD altered baseline proportions of cTFH, and whether this correlated with subsequent serological response to the full hepatitis B vaccination schedule (assay development and gating strategy shown in Fig 9). Absolute numbers of CXCR5+ memory CD4+ T cells (Fig. 9a), and the percentage of CXCR5+CD45RA- cells within the CD4+ T cell compartment were similar between CKD and HC at baseline (Fig. 10b). Further analysis showed that CKD patients demonstrated a significant reduction in CXCR3+CCR6- cells as a proportion of CXCR5+ memory T cells but not in CXCR3-CCR6- or CXCR3-CCR6+ compartments (Fig. 10c). Baseline CXCR5+ memory T cell compartments were then analyzed according to subsequent serological response to hepatitis B vaccine. CKD non-responders exhibited lower proportions of CXCR3+CCR6- cTFH at baseline compared to both CKD responders and HC non-responders (Fig. 10d).

Baseline PD-1 expression on cTFH was analyzed next, to assess pre-vaccination levels of cellular activation and exhaustion. The abundance of PD-1+ cells within CXCR5+ memory CD4+ T cells, and within each cTFH subset, was similar between HC and CKD (Fig. 11a, b), and between serological responders and non-responders (Fig. 11c). These findings contrast with previous reports that identified increased expression of activation and apoptosis markers on T cells in CKD.^{15,54} Age did not correlate with PD-1+ cTFH or PD-1+ cTFH subsets (*data not shown*).

Figure 9. TFH gating strategy. (opposite page) **a.** Staining and gating strategy for TFH cells was performed using human tonsil. After gating on each of the four populations within the CXCR5 CD45RA plot, cells were analyzed for CXCR3 versus CCR6 expression, and CCR7 versus PD-1 expression to identify the population of interest. CXCR5^{hi} PD-1^{hi} TFH cells are rare in peripheral blood (red square). The cells most closely resembling circulating counterparts of TFH in tonsil are CXCR5+PD-1+ cells (blue square), and therefore peripheral blood staining was based on this phenotype. **b.** Gating strategy performed on peripheral blood to determine CXCR5+ memory cells, subsets based on CXCR3 and CCR6 expression, and PD-1 expression. This was compared to CXCR3, CCR6 and PD-1 expression on CXCR5-CD45RA+ naive CD4+ T cells. After gating on CD4+ T cells, CXCR5+ memory cells (pink) and naive cells (orange) were then analyzed for CXCR3 and CCR6 expression. Most naive CD4+ T cells are CXCR3-CCR6- CCR7+, and do not express PD-1. Therefore, CXCR3-CCR6-naive cells were used to determine the PD-1 and CCR7 gates on CXCR5+ memory cell subsets, including for abundance of PD-1+ cells (histogram) irrespective of CCR7 expression. All values shown are percentages.



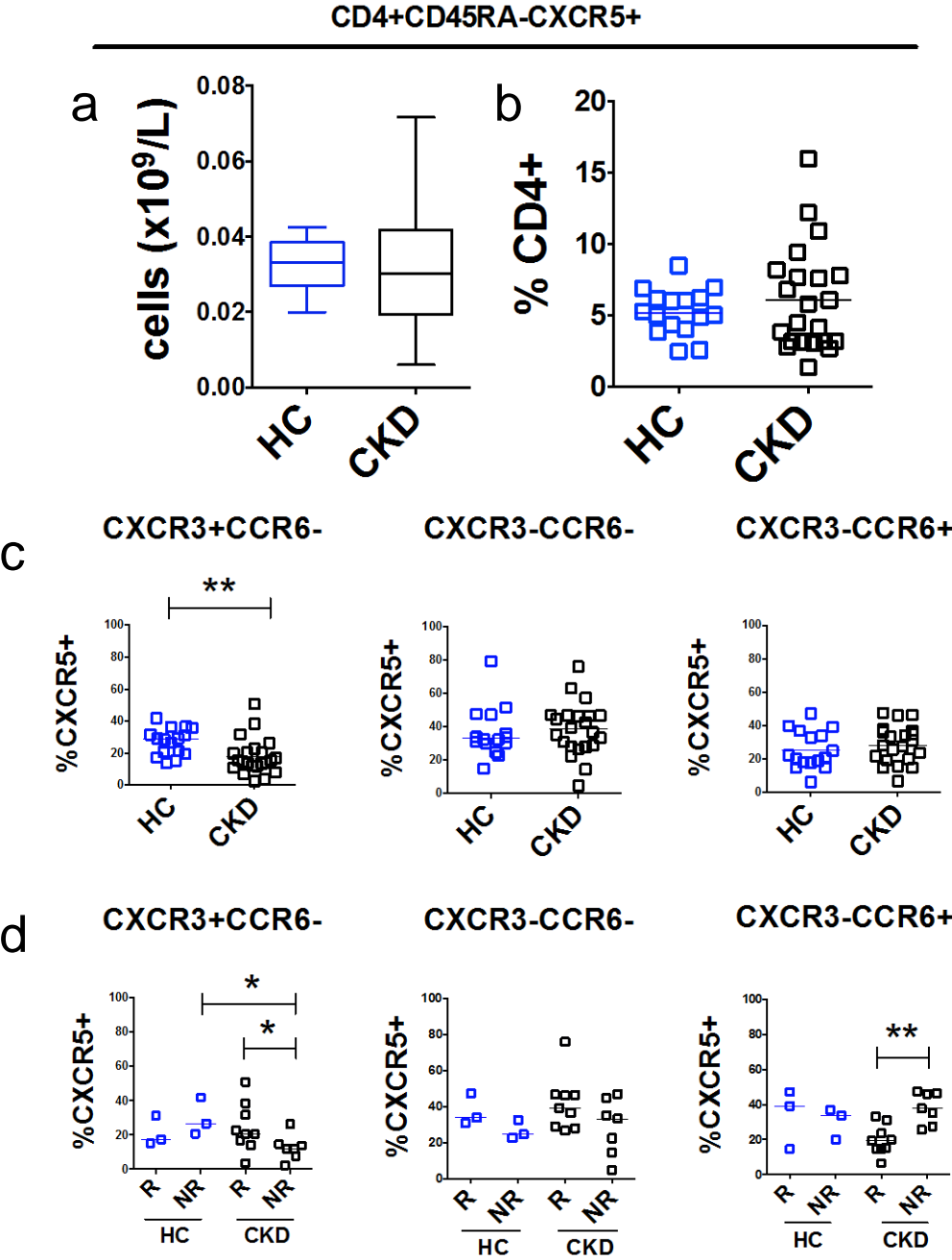
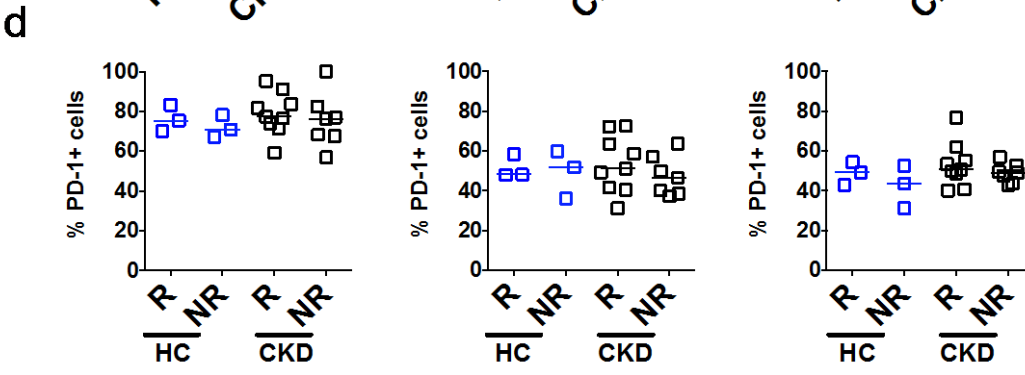
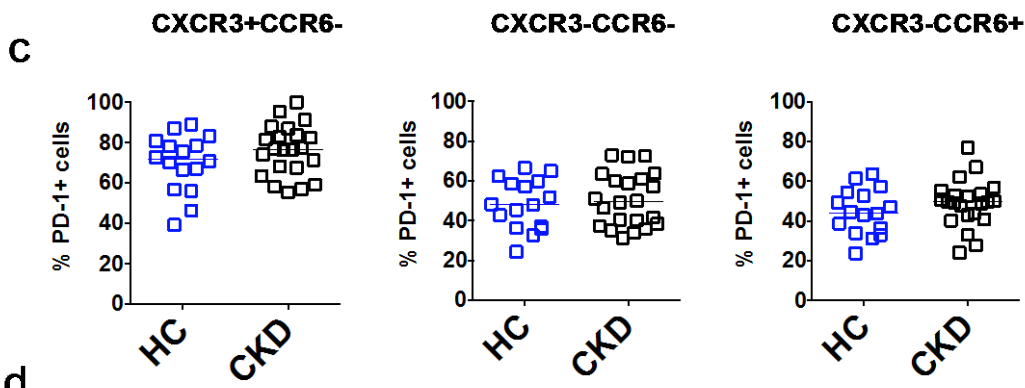
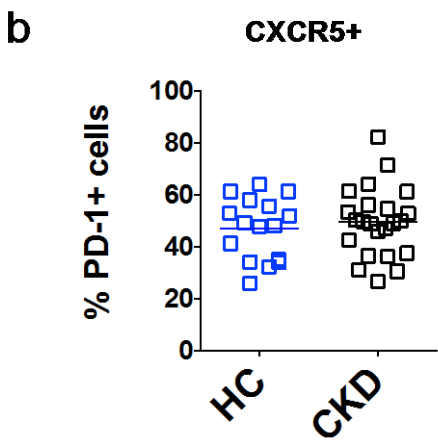
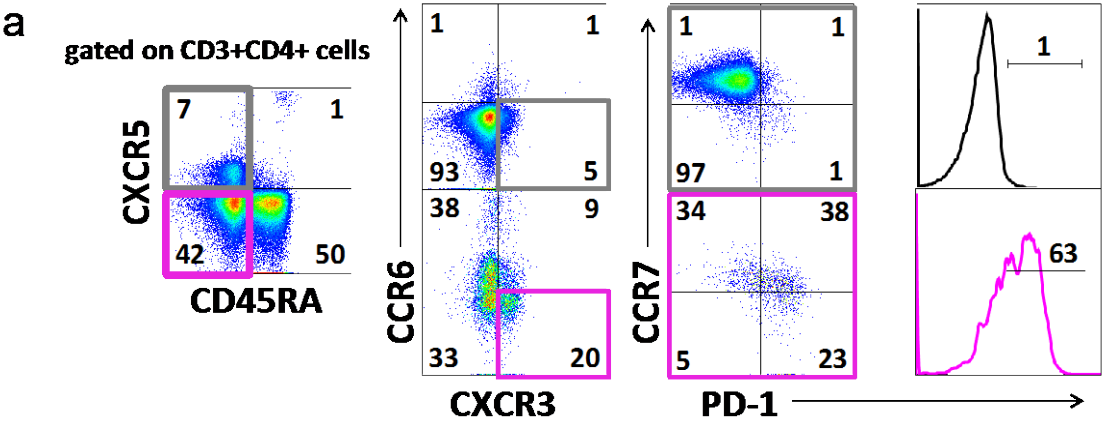


Figure 10. Baseline cTFH subsets in CKD and healthy controls (*opposite page*)

a. Baseline absolute numbers of CXCR5+memory CD4+ T cells in healthy controls (HC, n=16, blue) and CKD patients (n=24, black), including those who subsequently received either HBV or influenza vaccine. **b.** Baseline proportions of CXCR5+CD45RA- cells expressed as a percentage of CD4+ T cells, in healthy controls (HC, n=16) and CKD patients (n=24). **c.** Baseline cTFH subsets according to CXCR3 and CCR6 surface expression in healthy controls (HC, n=16) and CKD patients (n=24). **d.** Baseline cTFH subsets analyzed according to subsequent serological response (R, serological responder (HBsAb ≥ 10 mIU/ml); NR, serological non-responder (HBsAb < 10 mIU/ml)) in healthy controls (HC, n=6, blue) and CKD patients (n=19, black) who received HBV. Horizontal bars represent medians. Analysis performed using Mann-Whitney test of medians. * $p < 0.05$; ** $p < 0.01$.

Figure 11. Baseline expression of PD-1 on cTFH and cTFH subsets (*following page*).

a. Representative gating strategy for determining baseline PD-1 expression on cTFH subsets, here shown for CXCR3+CCR6- cTFH. PD-1 histogram gate was determined by using PD-1 negative CXCR5- memory CD4+ T cells. **b.** Baseline PD-1+ cells as a percentage of CXCR5+ memory CD4+ T cells. **c.** Baseline PD-1+ cells within each cTFH subset, and expressed as a percentage of that subset, in all healthy controls (blue squares, n=16) and all CKD patients (black squares, n=22). **d.** Baseline PD-1+ cells in cTFH subsets, displayed according to subsequent sero-responsiveness, in healthy controls (HC, blue squares) and CKD patients (CKD, black squares), who subsequently received hepatitis B vaccine and had post vaccination HBsAb measurement performed. Horizontal bars represent medians; analysis performed using Mann-Whitney test of medians. R – sero-responder (HBsAb ≥ 10 mIU/ml); NR – sero-non-responder (HBsAb < 10 mIU/ml).



3.1.7 Defective cTFH response in CKD

The T cell compartment was examined before and 7 days after vaccination. cTFH were defined as previously described⁴⁸ as CD4+CD45RA-CXCR5+CCR7^{lo} PD-1^{hi} (gating strategy shown in Fig. 12), and expressed as a proportion of memory T cells, or CXCR5⁺ memory T cells. There was no significant change in these parameters after either vaccination, in either HC or CKD, and no correlation with post vaccination HBsAb titres (*data not shown*).

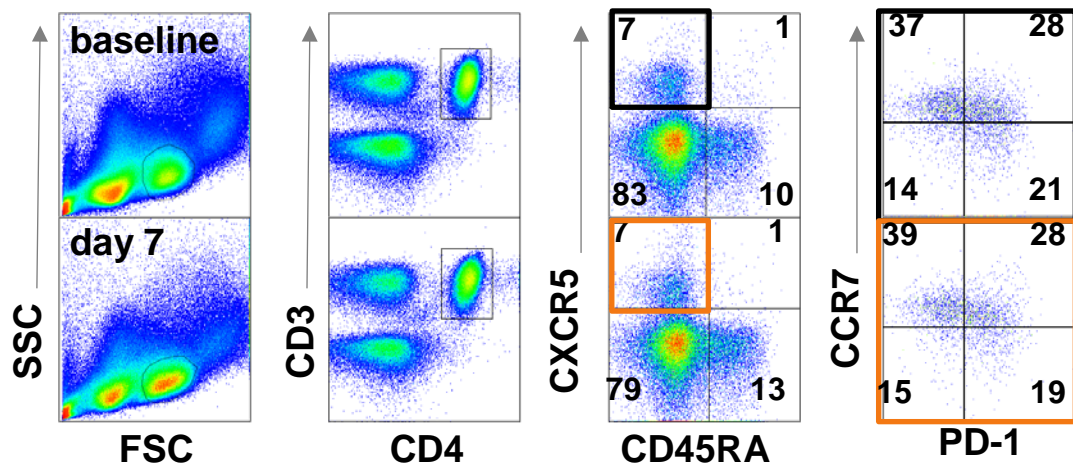


Figure 12. Identification of cTFH in peripheral blood.

Gating strategy for circulating TFH. After gating on forward and side scatter for lymphocytes, CD3+CD4+CD45RA-CXCR5+ cells were analyzed for expression of PD-1 and CCR7, on samples collected at baseline (before vaccination), and 7 days after vaccination. All values are percentages.

This compartment was analyzed further by looking for changes in PD-1 mean fluorescence intensity (Δ PD-1 MFI) on CXCR5+ memory T cells (gating strategy Fig. 13a). There was no significant change from baseline in either HC or CKD following HBV or seasonal influenza vaccine (Fig. 13b). When Δ PD-1 MFI on CXCR5+ memory cells was further analyzed according to CCR7 expression (gating strategy shown in Fig. 13c) a three-fold median increase in PD-1 expression on CCR7+ cells in HC after HBV was observed (Fig. 13e), but there was no consistent change in other vaccine groups. This increase was statistically significant when compared to the CKD 40 μ g vaccine group, but not the CKD 20 μ g vaccine group, or HC or CKD patients who had received seasonal influenza vaccine.

cTFH subsets have been demonstrated to undergo dynamic changes after influenza vaccination.^{42,43} The abundance and activation of each of these subsets was therefore examined before and after vaccination. In this cohort, neither HBV nor seasonal influenza vaccine elicited changes in the proportions of these subsets (Fig. 14). By contrast, there was a significant increase in PD-1 expression (as assessed by PD-1 MFI) on both CCR7- and CCR7+ cells within CXCR3+CCR6- cTFH (gating strategy Fig. 15a) after HBV in HC, but not in CKD (Fig. 15b and c). This did not, however, correlate with subsequent seroconversion or HBsAb titre in HC (*data not shown*). In order to determine whether absence of PD-1 upregulation on CXCR3+CCR6- cTFH cells was specific to HBV or to CKD, a similar analysis was performed after seasonal influenza vaccine. Interestingly, seasonal influenza vaccination elicited median increases in PD-1 MFI on both CCR7- and CCR7+ subsets, specifically in CXCR3+CCR6- cTFH, and the magnitude of increase was greater within the CCR7- subset. The responses were similar for magnitude and rate in both CKD and HC.

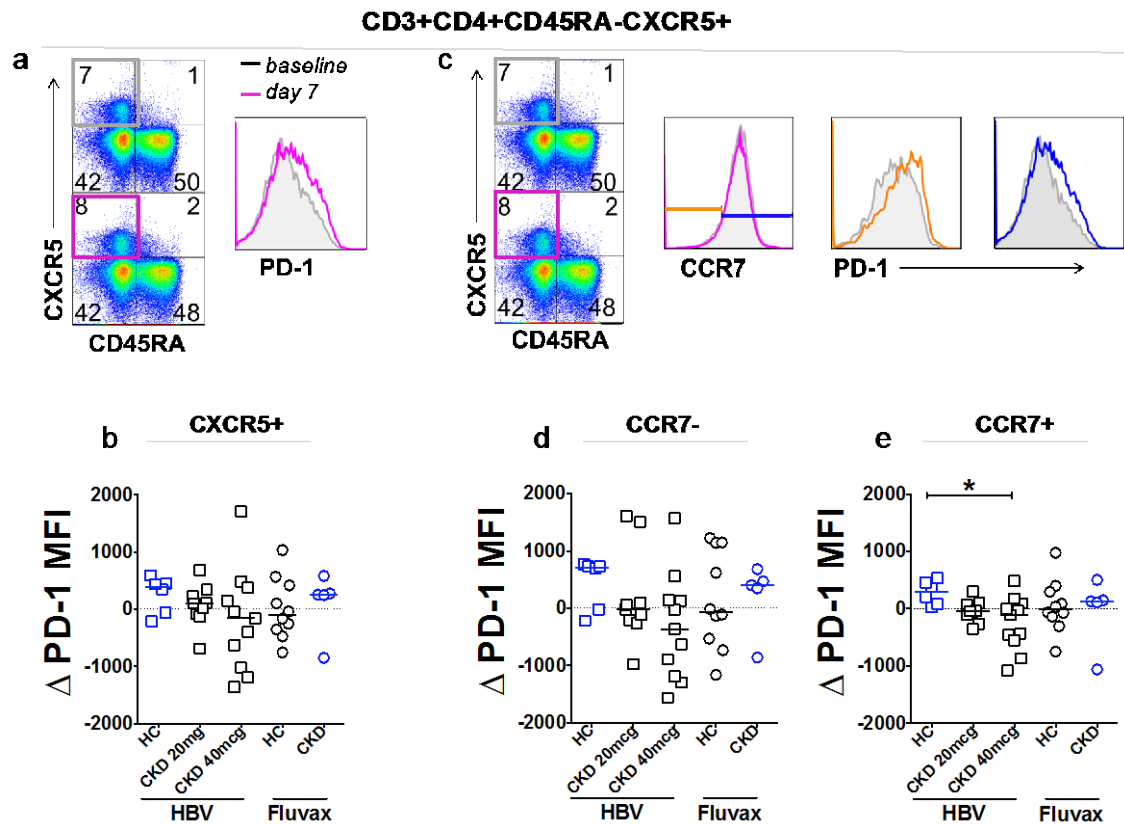


Figure 13. Vaccine induced change in PD-1 expression on CXCR5+ cTFH, and CCR7+ and CCR7- cTFH

a. Gating strategy to determine PD-1 MFI on CXCR5+ memory CD4+ T cells before and after vaccination. **b.** Change in PD-1 MFI on CXCR5+ memory CD4+ T cells after vaccination according to vaccine group. **c.** Gating strategy to determine PD-1 MFI according to CCR7 expression in CXCR5+ memory CD4+ T cells. Baseline, grey; Day 7, cerise; CCR7-, d7, orange; CCR7+ d7, blue. **d-e.** Vaccine-induced change in PD-1 MFI on **(d)** CCR7- and **(e)** CCR7+ compartments according to vaccine group. CKD, HBV (blue squares, 20mcg n=9, 40mcg n=11); HC, HBV (n=6, blue circles); CKD, Fluvax (blue circles, n=5); HC, Fluvax (black circles, n=10). Analysis performed using Mann-Whitney t-test, horizontal bars represent medians; *p=0.0120.

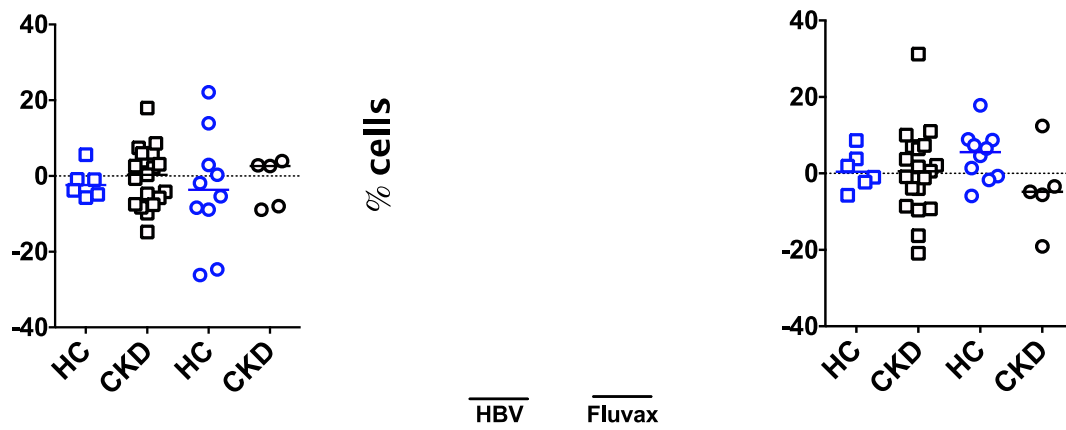
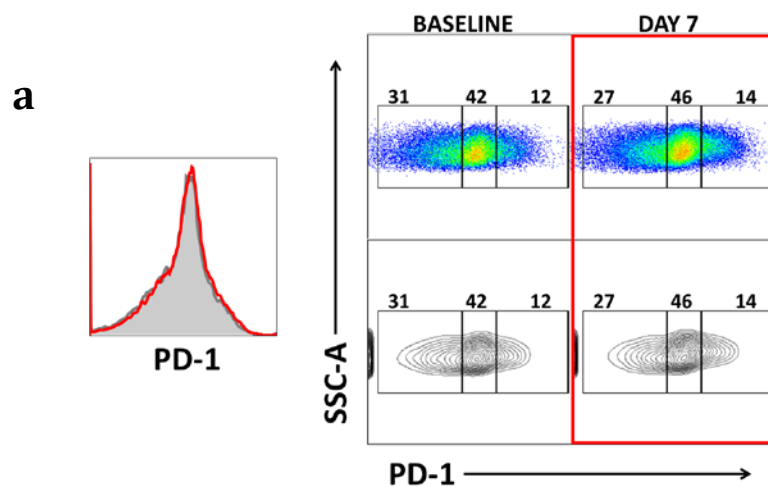


Figure 14. Abundance of TFH after vaccination

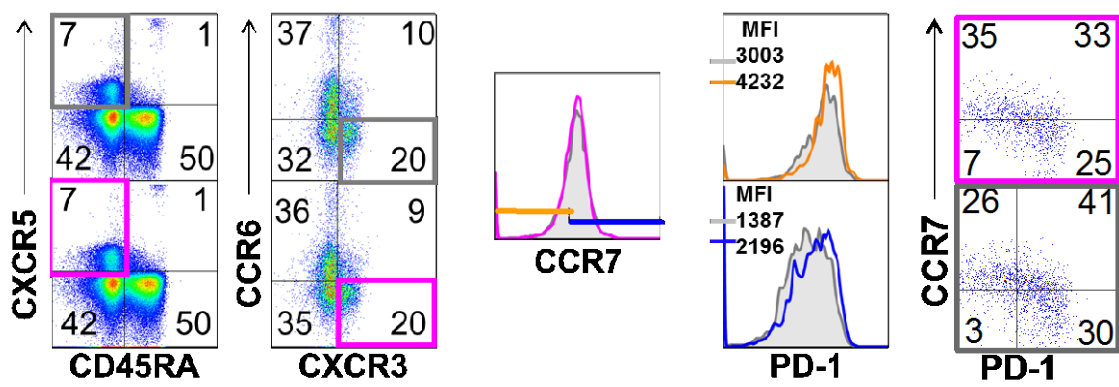
Change (Δ) in TFH subsets (cells as a % of CXCR5+ cells) after vaccination, according to vaccine type and clinical group. HC – healthy controls; CKD – chronic kidney disease; Fluvax – seasonal influenza vaccination; HBV – hepatitis B vaccine. Horizontal bars represent medians.

Figure 15. PD-1 is upregulated on CXCR3+CCR6- cTFH after vaccination, but not after HBV in CKD patients (*below and next page*)

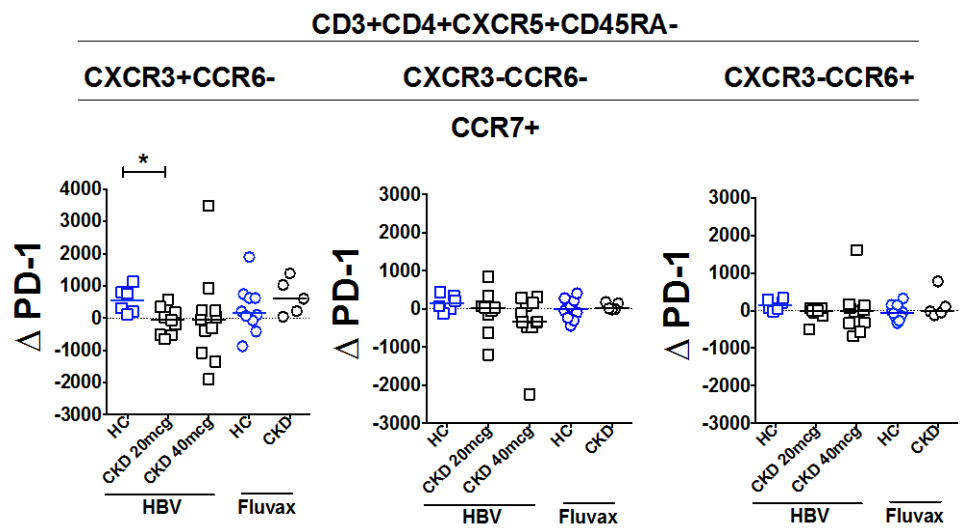
a. PD-1 expression on total lymphocytes at baseline (grey) and day 7 (red), with representative plots showing PD-1 histogram and colour dot plots. Similar to human tonsil, PD-1 expression on total lymphocytes has been divided into PD-1 negative, PD-1+ and PD-1 hi. **b.** Gating strategy to determine PD-1 MFI on CXCR5+ memory CD4+ T cells before (grey box and histograms) and after (colored box and histogram) vaccination, according to CXCR3 and CCR6 expression (CXCR3+CCR6- compartment shown here), followed by CCR7 expression. **c-d.** Change in PD-1 MFI in CCR7+ (**c**) and CCR7- (**d**) cTFH compartments after vaccination. HC who received Fluvax (n=10, blue circles) or HBV (n=6, blue squares). CKD patients who received HBV (20mcg n=9, 40mcg n=11; black squares) or Fluvax (black circles, n=5). Horizontal bars represent medians. *p<0.05. HBV, hepatitis B vaccine.



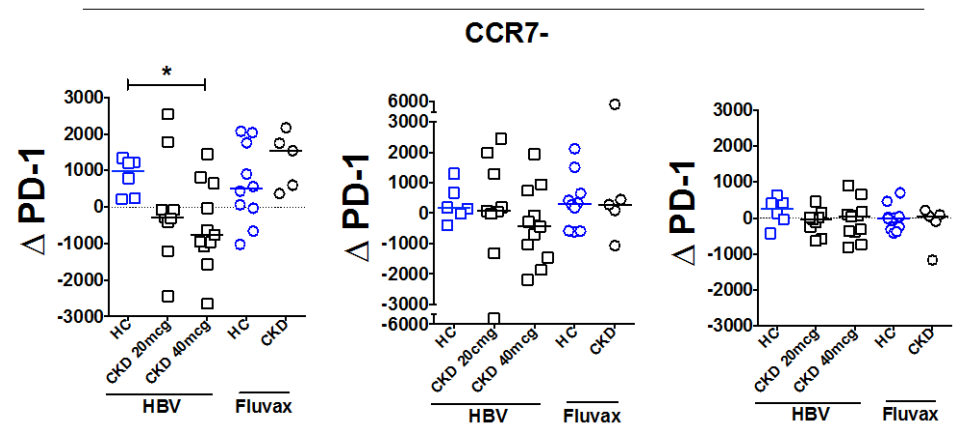
b



c



d



3.1.8 Intact plasmablast response in CKD

One possible explanation for the poor sero-response to HBV in CKD would be differences in PB formation (assay development and gating strategy shown in Fig. 16a-b). 55% of circulating PB expressed IgA at baseline, whereas 27% expressed IgG (Fig. 16c). PB were enumerated at baseline and day 7 (Fig. 17a). PB were characterized according to isotype, using cytoplasmic staining for IgG, IgA and IgM (Fig. 17b). The change in abundance of PB (or delta PB), was determined by comparing PB enumeration on day 7 to baseline. This analysis was performed for total PB as well as PB expressing each isotype. HBV did not elicit a PB response, nor a change in isotype, within the PB population, in either HC or CKD (Fig. 17c), including when accounting for HBV vaccine dose in CKD (Fig.17d). There was no correlation between PB response and either seroconversion or HBsAb titre after HBV, in either HC or CKD (Fig. 18).

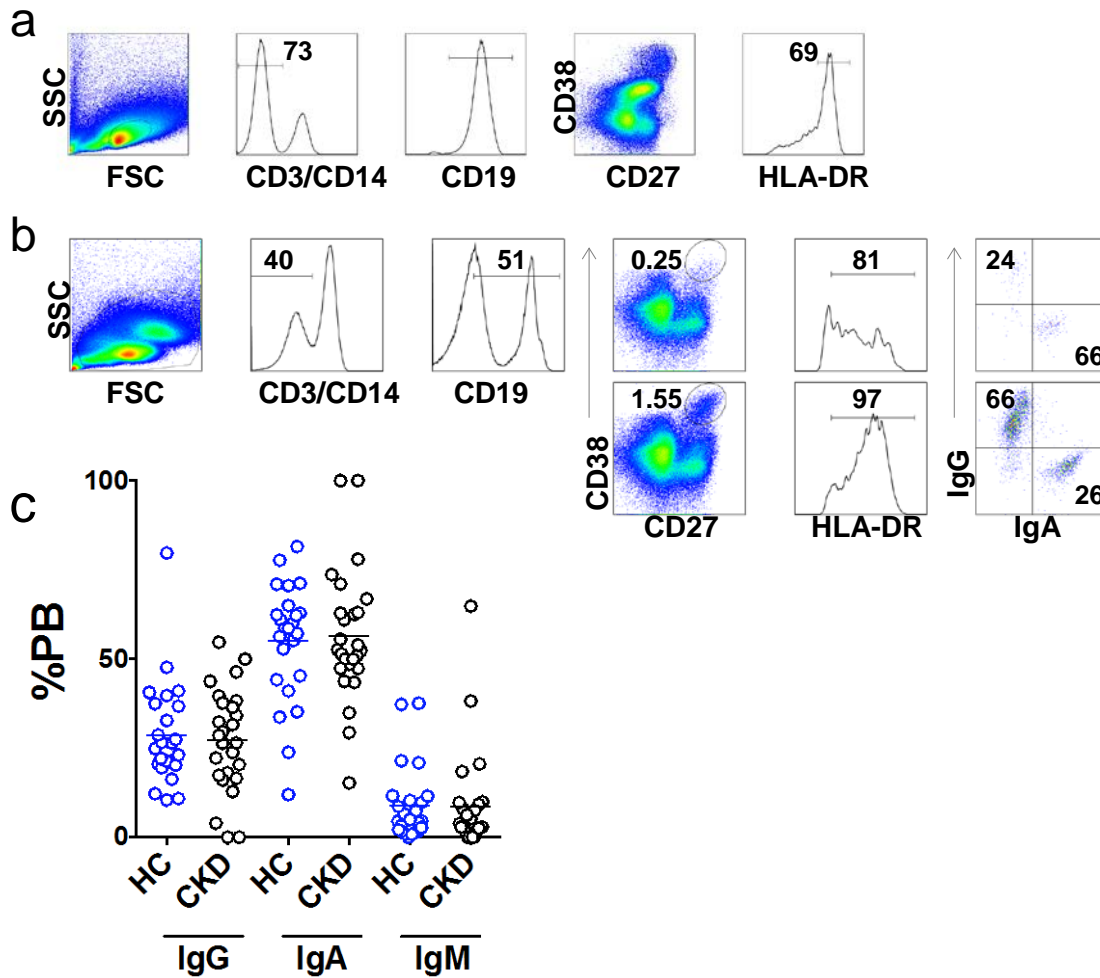


Figure 16. Plasmablast (PB) identification. a. Flow cytometric analysis of human tonsil for the identification of PB. After gating on lymphocytes, cell populations are selected which are negative for CD3 and CD14 expression, positive for CD19, CD27 and CD38 (oval gate), and express high levels of MHC class II. b. The same strategy is applied to the analysis of peripheral blood. Since PB downregulate CD19, this gate is set to capture CD19^{lo} cells. Analysis before (*top row*) and seven days after (*bottom row*) seasonal influenza vaccination in a healthy control identifies an increase in PB at day 7, which predominantly express cytoplasmic IgG. c. Baseline (prevaccination) proportions of plasmablasts according to cytoplasmic immunoglobulin (Ig) isotype expression in healthy controls (HC-blue circles) and CKD patients (black circles). Horizontal bars are medians. PB – plasmablasts.

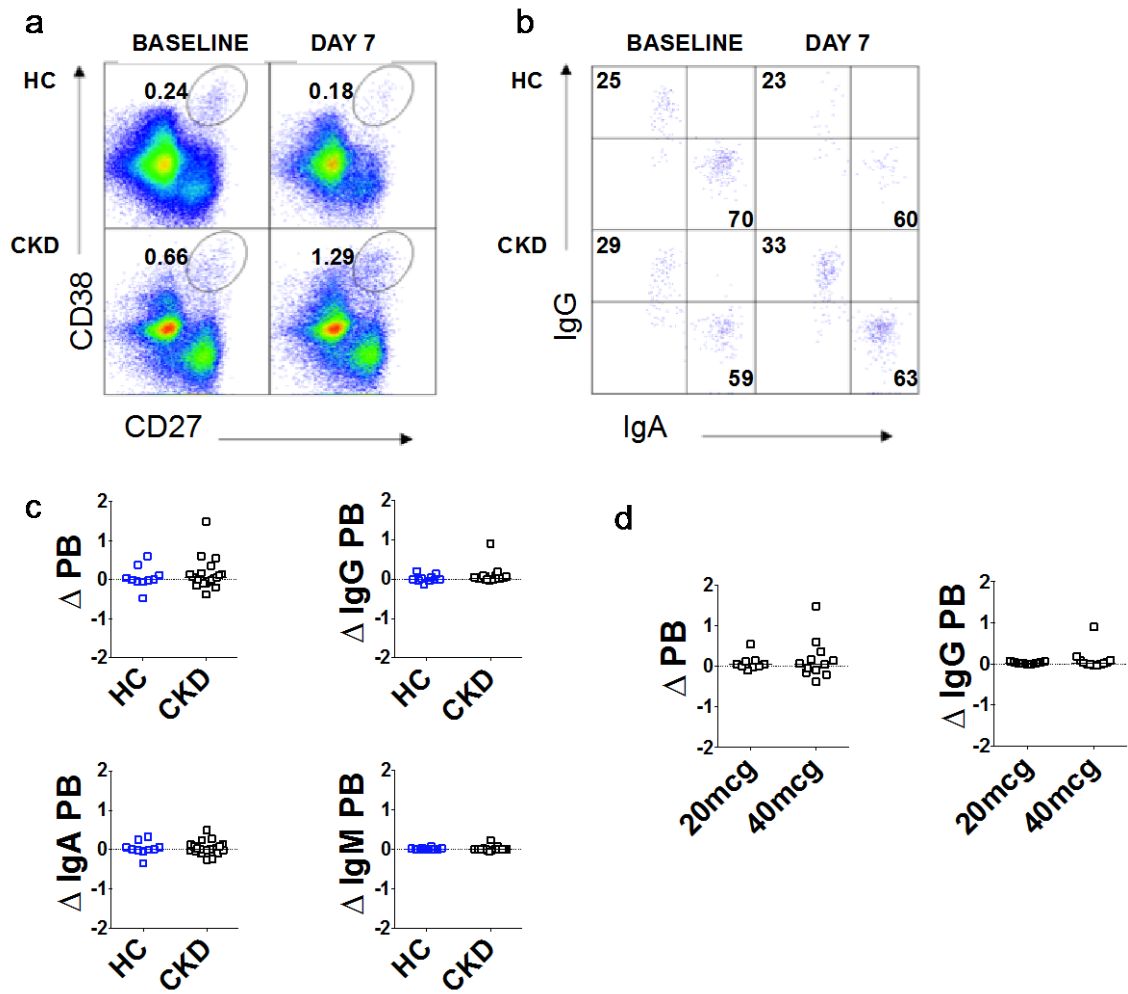


Figure 17. Plasmablasts analysis following HBV

a-b. Representative FACS analysis for plasmablast enumeration (**a**) and isotyping (**b**)

before and 7 days after recombinant hepatitis B surface antigen vaccination. **c.**

Plasmablast responses for total and isotyped PB. **d.** Total and IgG plasmablast responses

according to HBV dose schedule (20mcg or 40mcg schedule) in CKD patients. HC, healthy

controls (blue), n=10; CKD, chronic kidney disease patients (black), n=21; PB,

plasmablasts; Δ = change in PB (day 7 minus PB at baseline, when expressed as a

percentage of total B cells).

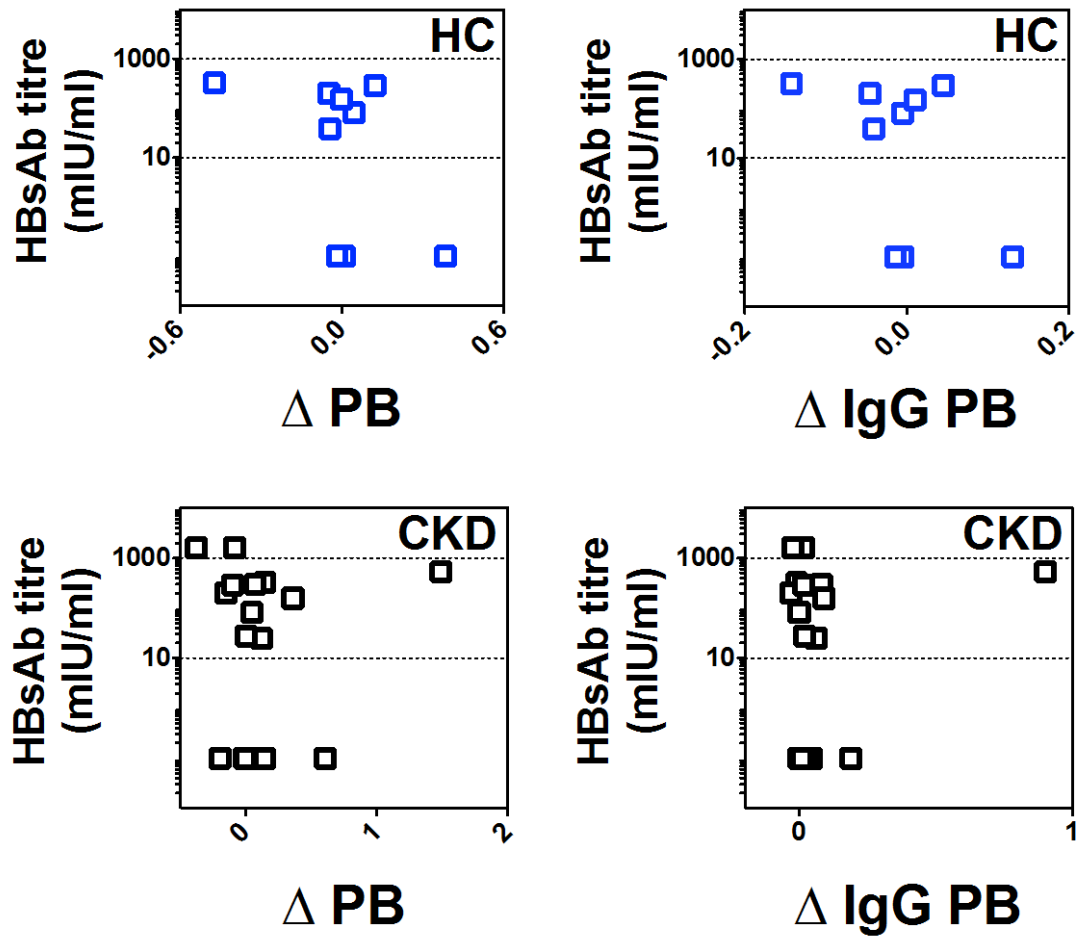


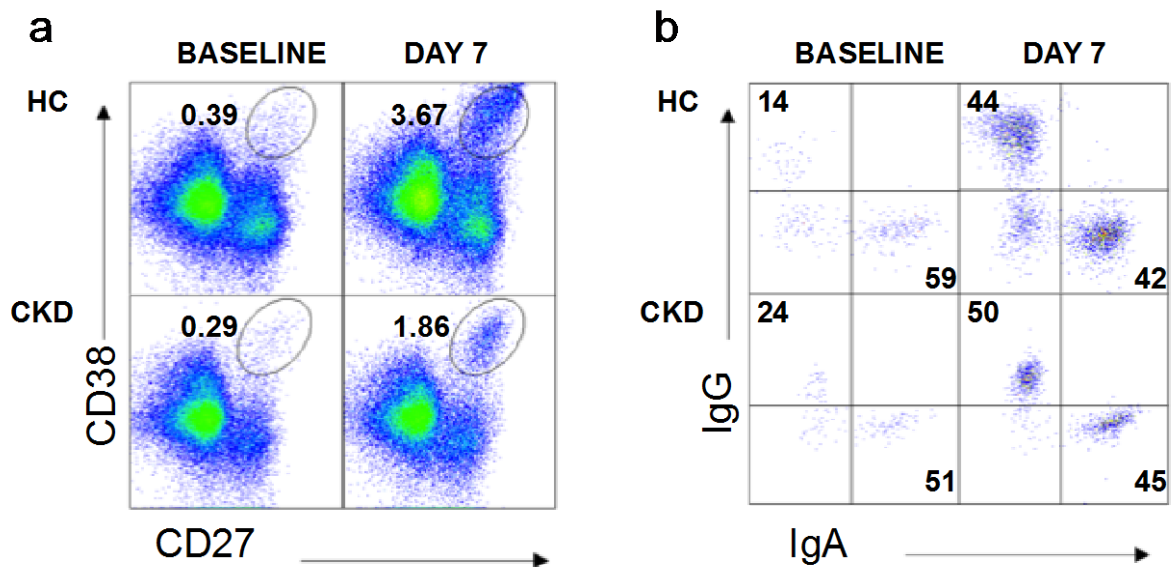
Figure 18. Relationship between plasmablast response and post-vaccination

HBsAb titre. Healthy controls (HC, blue squares) and CKD patients (CKD, black squares); Δ PB - change in total plasmablasts expressed as a percentage of total B cells; (Δ IgG PB) change in IgG plasmablasts expressed as a percentage of total B cells. Dashed horizontal lines represent upper and lower limits of detection of HBsAb assay. HBsAb – hepatitis B surface antibody. Statistical analyses performed using Spearman test of correlation.

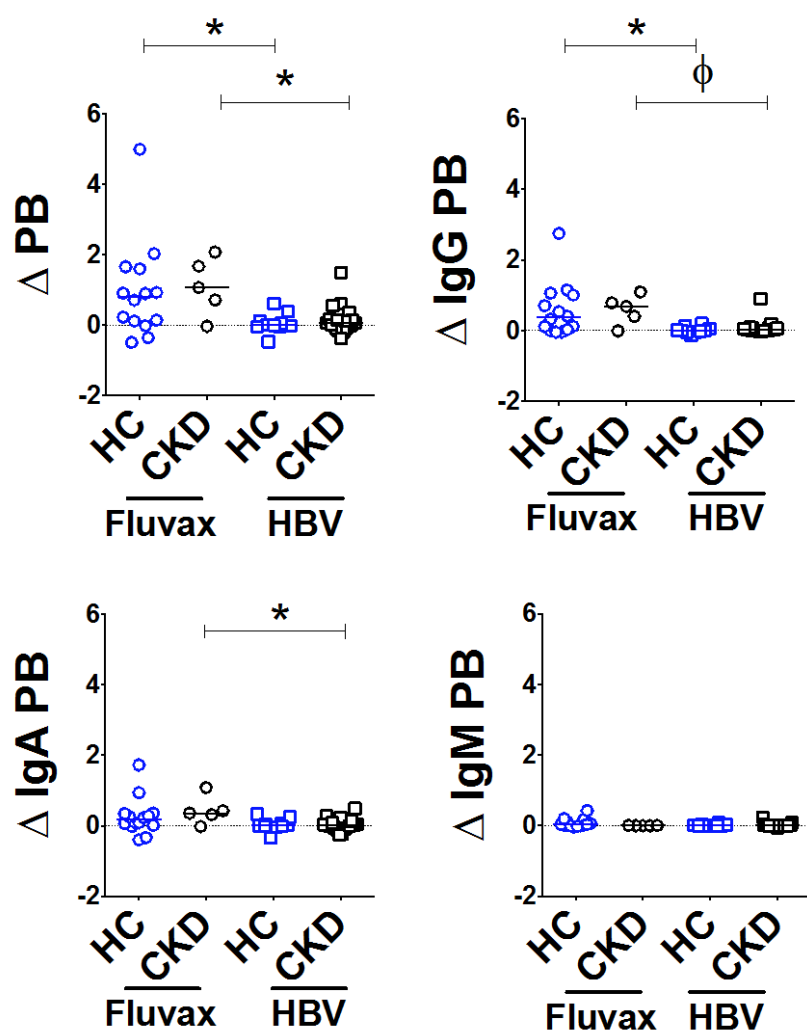
Within the CKD cohort, there was no correlation between eGFR and PB response (*data not shown*). To clarify whether the absence of a PB response was specific to HBV, PB responses to seasonal influenza vaccine were compared in HC and CKD. By contrast, seasonal influenza vaccine elicited a robust PB response of similar magnitude in both HC and CKD (Fig. 19a), predominantly accounted for by an increase in IgG PB, with a smaller increase in IgA PB (Fig. 19b). The change in total PB, and IgG PB, was significantly greater after seasonal influenza vaccination than after HBV in HC (Fig. 19c). A similar difference between vaccination types was seen in CKD, with increases in total and IgA PB significantly greater after seasonal influenza vaccination.

Figure 19. (below and opposite page) Plasmablast response after seasonal influenza vaccine

a-b. Representative FACS analysis for plasmablast enumeration (**a**) and isotyping (**b**) before and 7 days after Fluvax. **c.** Plasmablast responses for total and isotyped PB, following either Fluvax (circles) or HBV (squares), in either healthy controls (HC, blue) or CKD patients (black). Horizontal bars represent medians. * $p < 0.05$, ϕ $p = 0.0510$. Analyses performed using Mann-Whitney test. HC, healthy controls; CKD, chronic kidney disease; PB, plasmablasts; Δ PB, change in plasmablasts, from baseline to day 7, expressed as a percentage of B cells.



C



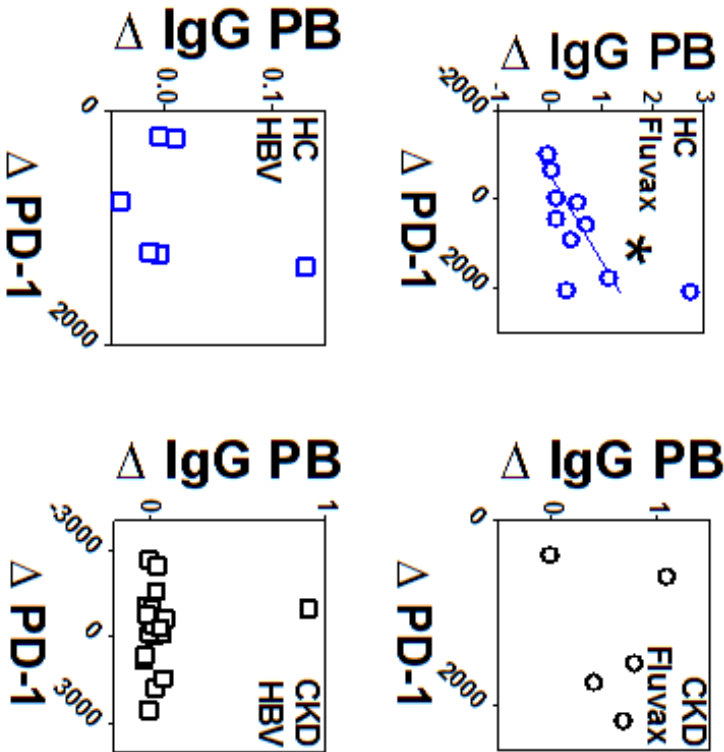
3.1.9 PB response correlates with PD-1 upregulation on CXCR3+ cTFH in HC

This study has demonstrated that HBV elicits upregulation of PD-1 on CXCR3+ CCR6- cTFH in HC, but not in CKD. By comparison, seasonal influenza vaccine elicits both upregulation of PD-1 on CXCR3+CCR6- cTFH and a PB response, in both HC and CKD. It was of interest therefore whether there might be a correlation between cTFH and PB responses. There was a strong correlation between IgG PB response and PD-1 upregulation within CXCR3+CCR6-CCR7- cTFH and CXCR3+CCR6-CCR7+ cTFH in HC who received seasonal influenza vaccine (Fig. 20a and 20b), but not in CKD after seasonal influenza vaccine, or after HBV in any group. Significant correlations were also seen between IgG PB response and CXCR5+ cTFH (Fig. 21a), as well as total CCR7- and CCR7+ cTFH (Fig. 21b and c). Again no such correlations were seen in CKD, or after HBV in any vaccine group.

Figure 20. (opposite page) Upregulation of PD-1 on CXCR3+CCR6-CXCR5+ cTFH after Fluvax in healthy controls. a-b. Relation between IgG PB plasmablast responses and either CCR7- (**a**) or CCR7+ (**b**) CXCR3+ CCR6- CD4+ cTFH cells in healthy controls (HC, blue) or CKD (black) after either Fluvax (circles, upper panels) or HBV (squares, lower panels). Δ IgG PB, change in IgG plasmablasts. Δ PD-1, change in mean fluorescence intensity of PD-1. Horizontal bars represent medians. All analyses performed using Mann-Whitney test. Correlation analyses performed using Spearman test. * $p=0.0105$ and $R^2=0.7818$; ** $p=0.0010$ and $R^2=0.8396$.

CXCR3+CCR6-CCR7-

a



CXCR3+CCR6-CCR7+

b

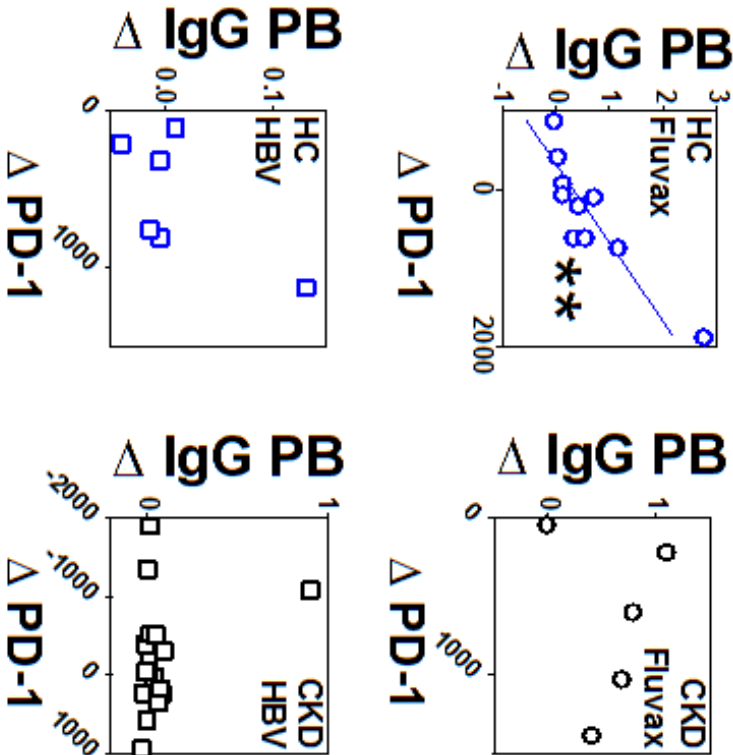


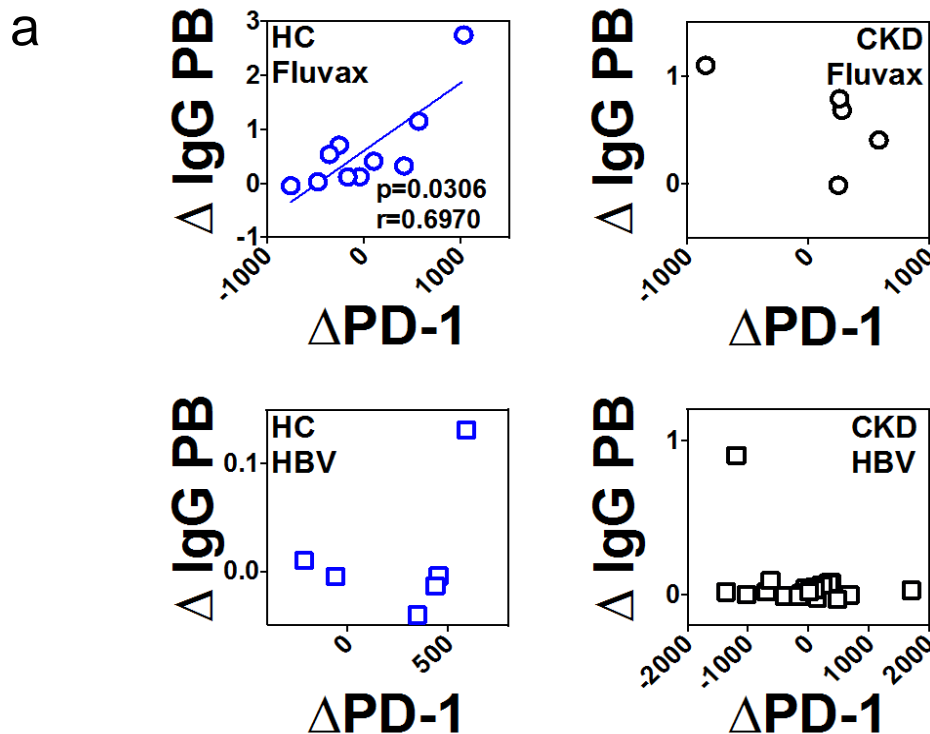
Figure 21. (below and opposite page) Correlation between the vaccine-induced change in circulating IgG plasmablasts

Examination of the relation between change in plasmablasts (Δ IgG PB) and the change in PD-1 MFI (Δ PD-1) on (a) cTFH, (b) CCR7+ cTFH and (c) CCR7- TFH subsets, according to vaccine group. HC – healthy controls; CKD – chronic kidney disease; Fluvax - seasonal influenza vaccine; HBV - hepatitis B vaccine; HBsAb, -hepatitis B surface antibody.

Correlation analysis performed using Spearman test. * $p=0.0174$ and $R^2=0.7455$;

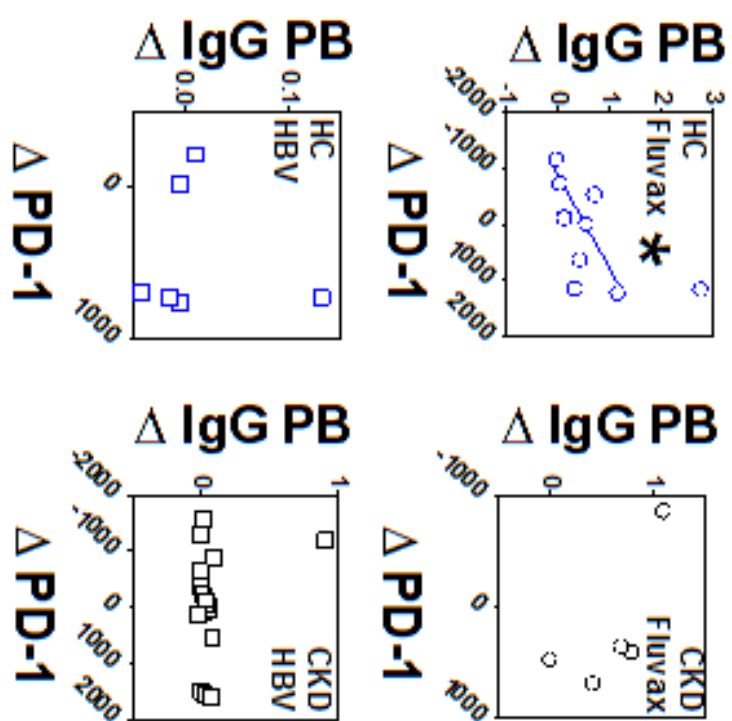
** $p=0.0029$ and $R^2=0.8545$.

CD3+CD4+CD45RA- CXCR5+



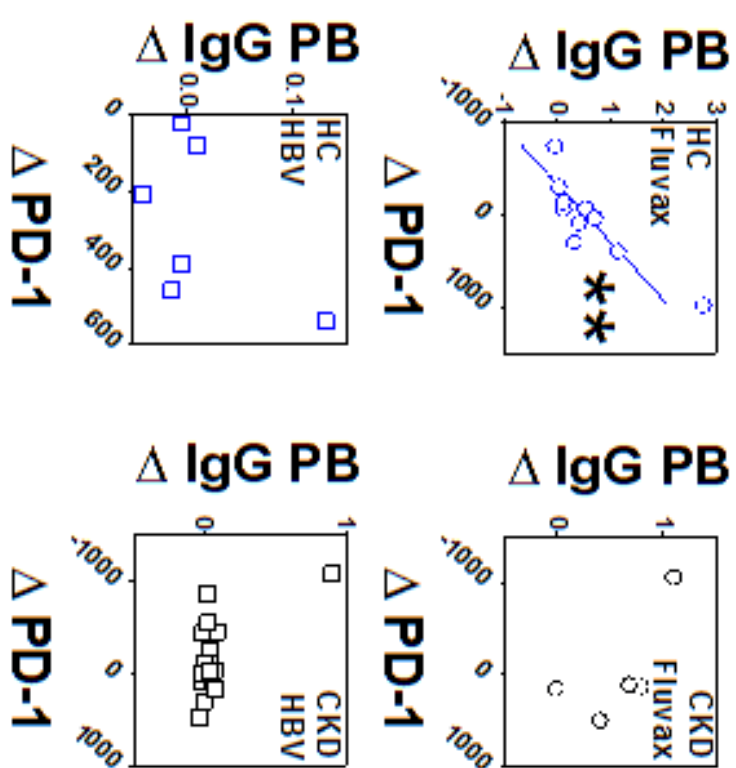
b

CCR7-



c

CCR7+



3.2 Haemodialysis cohort (2015-2016)

3.2.1 Enrolment

22 patients with end-stage renal failure already receiving regular haemodialysis were recruited to participate in the next phase of the study. 10 of these were recruited to receive a single 40µg HBV booster; 3 received the 2015 seasonal influenza vaccine; and 9 received the 2016 seasonal influenza vaccine. 22 healthy controls were also recruited during this period to receive the seasonal influenza vaccine only: 10 were vaccinated in 2015, and 12 were vaccinated in 2016. Patient characteristics are summarized in Table 3. The HBV HD group were significantly older than the HC group, but not the HD group who received the seasonal influenza vaccine.

Table 3: Patient demographics in the haemodialysis cohort (2015-2016) according to vaccine groups. HC – healthy controls; HD – haemodialysis. *Mann-Whitney t-test of medians. **when comparing HD HBV and HC.

	HD	HC	p value*
hepatitis B vaccine			
patients (n)	10	-	
age (median, years)	71.5	-	**<0.0001
age (range, years)	63-88	-	
male:female	1:1	-	
seasonal influenza vaccine			
patients (n)	12	22	
age (median, years)	62	**51	NS
age (range, years)	31-84	25-78	
male: female	3:1	3:8	

3.2.2 Post-vaccination HBsAb serology

Post-vaccination HBsAb titre was available for eight HD patients, of which five demonstrated a serological response (fig. 22). Only two patients achieved an HBsAb titre of >100mIU/ml, and none achieved a titre >1000mIU/ml. Post-vaccination HBsAb titre did not correlate with age. Two HD patients did not provide a day 28 serum sample for HBsAb titre determination, one due to palliation and discontinuation of the study, and the other due to cause unknown.

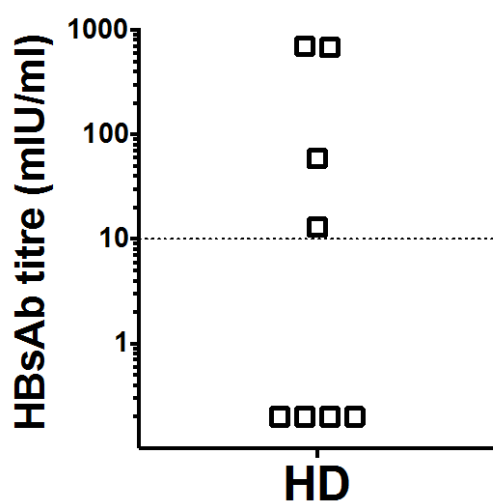


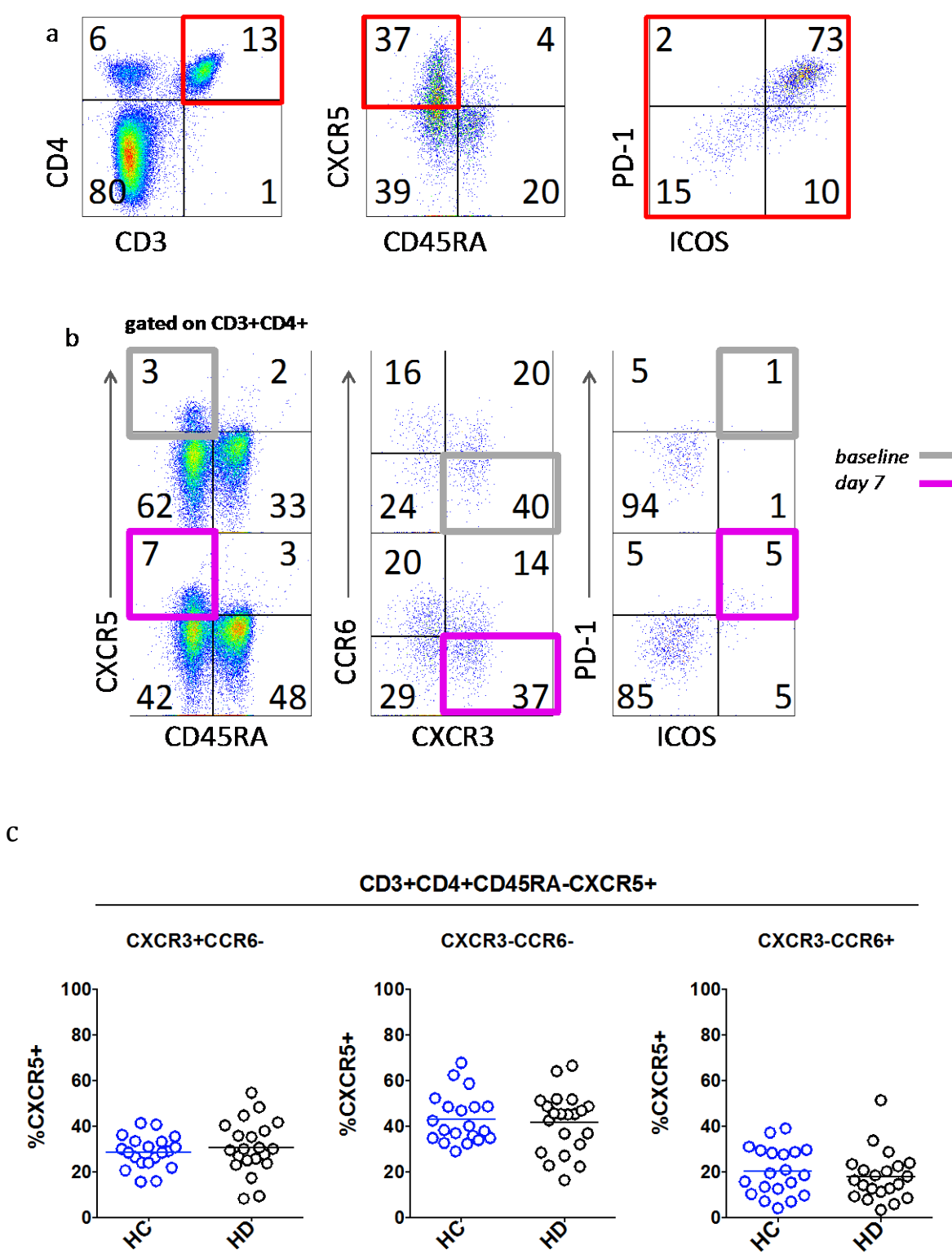
Figure 22. Day 28 post-vaccination hepatitis B surface antibody titre (HBsAb) in haemodialysis (HD) patients who had received hepatitis B booster vaccine (40µg). The horizontal dotted line represents the lower limit of detection of the assay (10mIU/ml). Undetected values are assigned an arbitrary value of 0.1mIU/ml.

3.2.3 Baseline cTFH analysis

The gating strategy for determination of cTFH and cTFH subsets according to CXCR3 and CCR6 expression was identical to that applied to the CKD cohort (gating strategy shown in Figure 23a and b) except for the exclusion of CCR7 and inclusion of ICOS. Overall, HD patients did not demonstrate elevated baseline cTFH subsets when compared to healthy controls (Figure 23c), suggesting that the reduced CXCR3+CCR6- cTFH subset seen at baseline in CKD is corrected by haemodialysis. Despite this, proportion of CXCR3+ cTFH did not predict subsequent seroresponse or HBsAb titre following HBV (data not shown). There was no difference in baseline expression of PD-1 or ICOS on cTFH or cTFH subsets between HC and HD.

Figure 23. (opposite page) a-c. Assessment of baseline cTFH in HD patients.

a. The gating strategy for PD-1 and ICOS was determined using normal human tonsil, which contains high numbers of germinal centre TFH, which express high levels of ICOS and PD-1. After gating on forward and side scatter, and exclusion of doublets, germinal centre TFH cells were identified as CD3+ CD4+, CXCR5+ CD45RA- cells, ICOS+ and PD-1+. This gate was used to set the gate on peripheral samples to determine expression of ICOS and PD-1 on cTFH. **b.** Gating strategy for ICOS and PD-1 expression on cTFH, using CXCR3+CCR6- cTFH as an example, performed at baseline and day 7 on a healthy control who had received the 2015 seasonal influenza vaccine. **c.** Baseline cTFH subsets in healthy controls (HC, blue circles, n=22) and haemodialysis patients (HD, black circles, n=22) using single platform analysis with flow cytometry, expressed as a percentage of CXCR5+ cTFH. Solid horizontal lines represent medians.

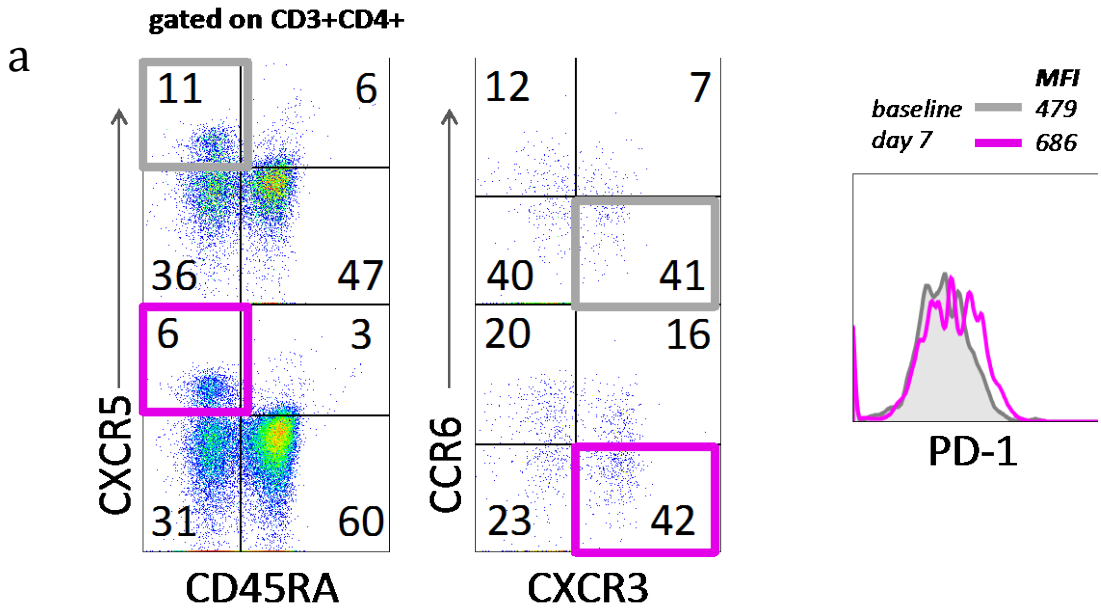


3.2.4 Upregulation of PD-1 on CXCR3+ cTFH

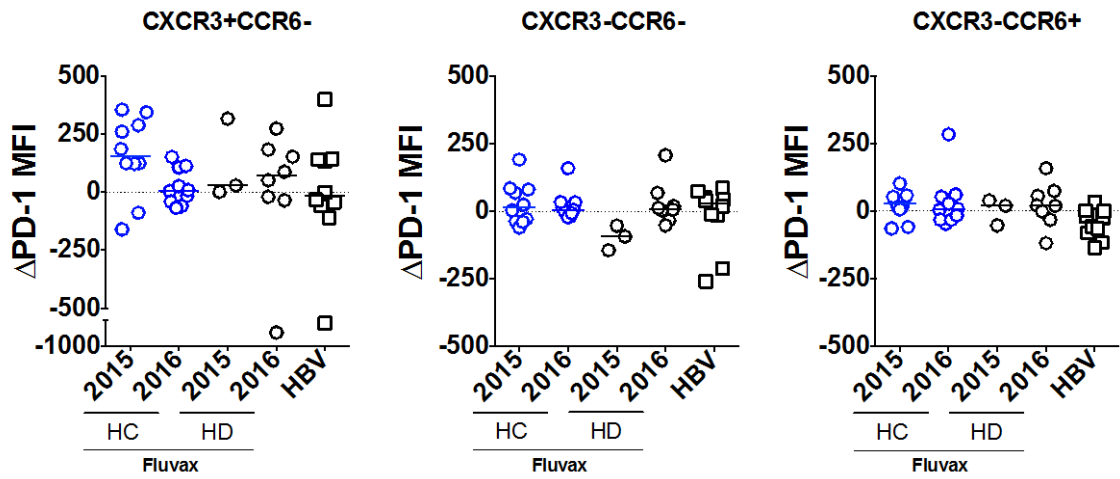
Paired cellular samples were available for all 22 HD patients, and 20 HC (10 had received 2015 seasonal influenza vaccine, and 10 had received 2016 seasonal influenza vaccine). The gating strategy to interrogate cTFH subset activation was similar to that applied to the CKD cohort except with the exclusion of staining for CCR7, and inclusion of staining for ICOS expression. As seen in the CKD cohort, no change in percentages of CXCR5+ cTFH or cTFH subsets was seen after either seasonal influenza vaccination in HC or HD, or after HBV in HD (*data not shown*). Upregulation of PD-1 on CXCR3+CCR6-cTFH, as measured by PD-1 MFI (gating strategy shown in Fig. 24a), was seen consistently only in healthy controls who had received the 2015 seasonal influenza vaccine, but not in other vaccine groups (Fig. 24b). No consistent change in PD-1 was seen in other cTFH subsets. There was no direct correlation between PD-1 upregulation on CXCR3+ cTFH and HBsAb in HD (Figure 24c). Of the four patients whose CXCR3+ cTFH demonstrated PD-1 upregulation, two seroconverted, one did not, and post-vaccination HBsAb titre was not available for one patient.

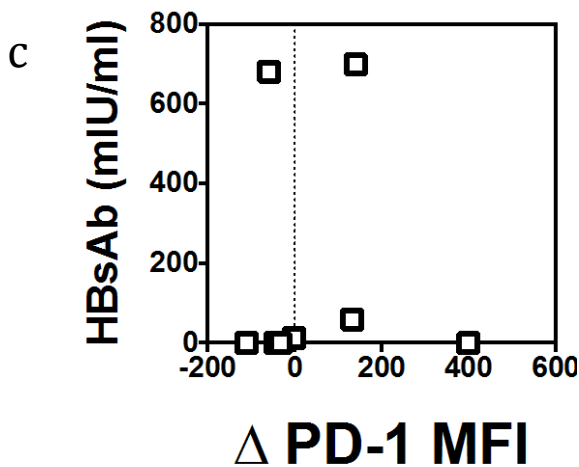
Figure 24. (opposite page) Change in PD-1 MFI on cTFH subsets after vaccination.

a. Representative gating strategy for PD-1 expression on cTFH subsets, measured by mean fluorescence intensity (MFI). **b.** Change in PD-1 MFI (Δ PD-1 MFI) on cTFH in healthy controls (HC) and haemodialysis patients (HD) according to vaccine type and year. **c.** Analysis of change in PD-1 MFI on CXCR3+ cTFH compared to day 28 post-vaccination HBsAb titre in HD. Fluvax – seasonal influenza vaccine; HBsAb – hepatitis B surface antibody; HBV – hepatitis B vaccine.



b





3.2.5 Induction of ICOS⁺ on cTFH correlates with HBsAb titre in HD

While PD-1 is a marker of cTFH activation, ICOS is a marker of cTFH capable of germinal centre reactions.^{37,47,55} There was no change in ICOS MFI on CXCR5⁺ cTFH subsets or cTFH (*data not shown*). There was, however, an increase in PD-1-ICOS⁺ cells within CXCR3⁺CCR6⁻ cTFH, seen after seasonal influenza in both HC and HD (fig. 25a), but not within CXCR3⁺CCR6⁻ or CXCR3⁺CCR6⁺ cTFH compartments. While a consistent increase in PD-1-ICOS⁺ cells within CXCR3⁺CCR6⁻ cTFH was not seen after HBV in HD, this parameter correlated with day 28 post-vaccination HBsAb titre (fig. 25b).

3.2.6 Induction of ICOS⁺ on cTFH does not correlate with PB response

Seasonal influenza vaccination elicited a PB response in some, but not all, HC and HD, such that no consistent PB response was seen (fig.26). In those who did demonstrate a PB response, this was accounted for by an increase in IgA PB, not IgG PB. No IgG PB response was seen at all. However, there was no correlation between the induction of ICOS⁺ on cTFH and PB, when analyzed according to total, IgA or IgG PB (*data not shown*). There was also no correlation between PB

response, of any isotype, and changes in PD-1 MFI on CXCR3+ cTFH, including in the 2016 HC group who appears to have overall increases in both parameters (data not shown).

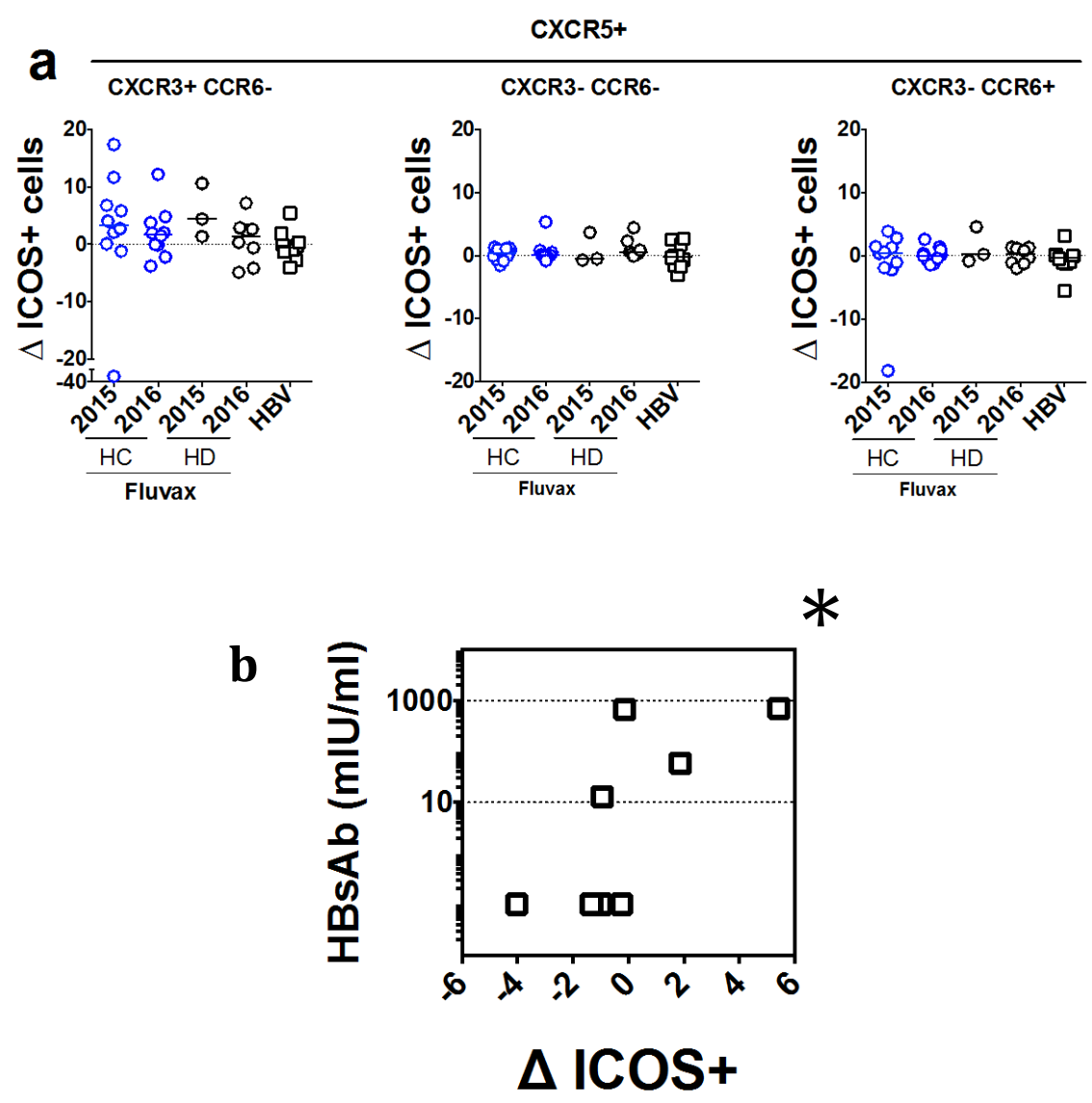


Figure 25. An increase in ICOS+ cells is specific to the CXCR3+ cTFH compartment. a. Change in abundance of ICOS+ cells within cTFH compartments after vaccination in

healthy controls (HC) and haemodialysis patients (HD) according to vaccination type and year. **b.** Change in abundance of ICOS⁺ cells within CXCR3⁺ cTFH correlates with day 28 post-vaccination HBsAb in HD after booster HBV. Spearman test of correlation. * $p = 0.0143$. Δ ICOS⁺ - change in ICOS⁺ cells within CXCR3⁺ cTFH. Fluvax – seasonal influenza vaccine; HBsAb – hepatitis B surface antibody; HBV – hepatitis B vaccine.

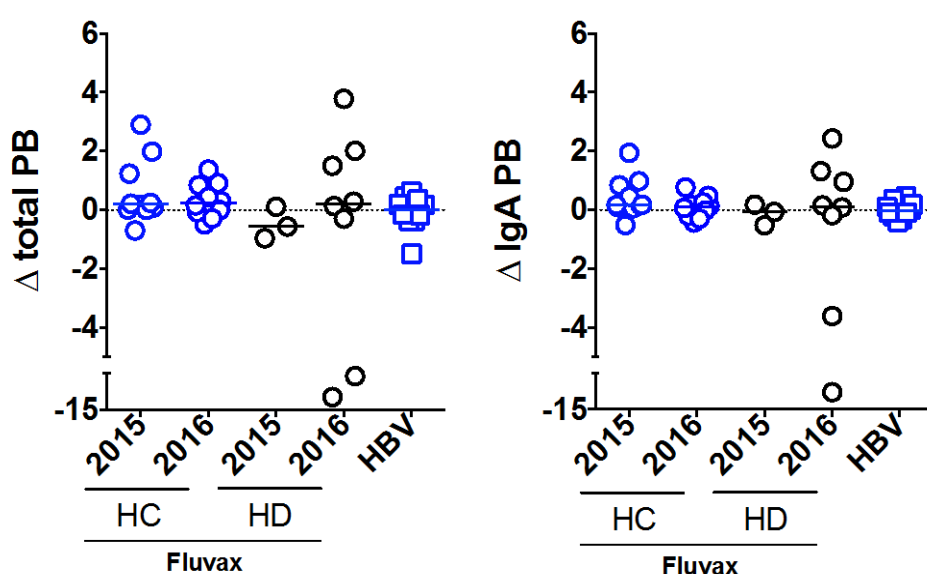
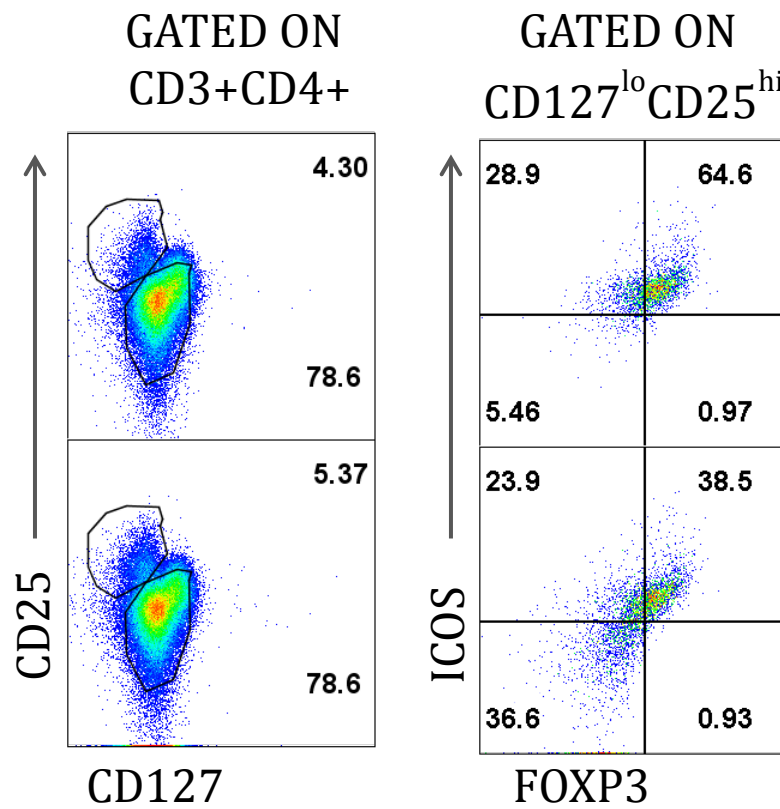


Figure 26. (above). Plasmablast response to vaccination in haemodialysis. The change in plasmablasts, calculated as the difference in plasmablast numbers at day 7 compared to baseline, and expressed as a percentage of B cells, after vaccination in healthy controls (HC) and haemodialysis patients (HD) according to vaccine type. Δ total PB – change in total plasmablasts; Δ IgA PB – change in IgA plasmablasts; Fluvax – seasonal influenza vaccine; HBV – hepatitis B vaccine.

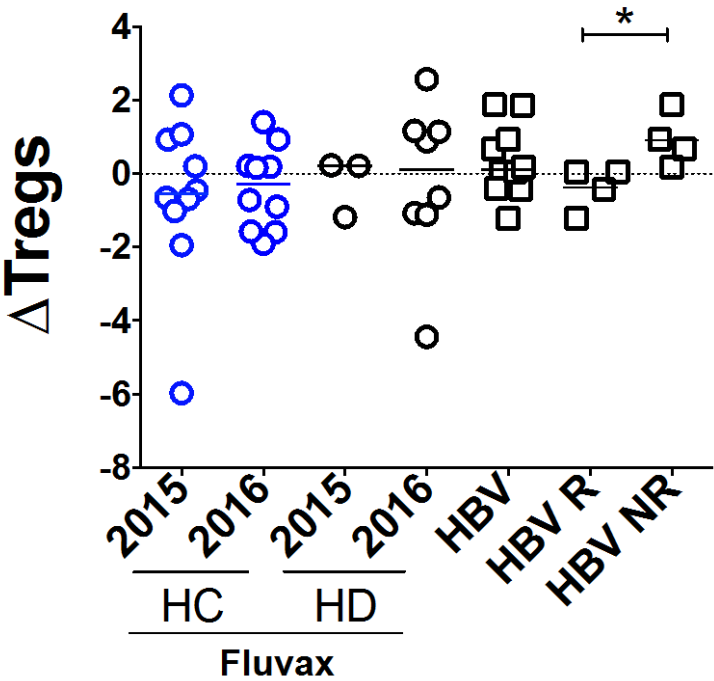
Figure 27. (opposite page). Change in abundance of regulatory T cells after vaccination. a. Gating strategy to determine Tregs and ICOS expression at baseline (top row) and day 7 (bottom row) after vaccination. After gating on lymphocytes, followed by selection of CD3⁺CD4⁺ cells, Tregs were identified by high expression of CD25, low

expression of CD127, and high expression of FOXP3. These cells were then analyzed for ICOS and PD-1 expression. **b.** The change in Tregs, expressed as a percentage of CD3+ T cells, after vaccination in healthy controls (HC) and haemodialysis patients (HD) according to vaccine type and year, and seroresponse to HBV in HD. Δ Tregs – change (delta) in regulatory T cells; HBV – hepatitis B vaccine; HBV R – hepatitis B vaccine seroresponders; HBV NR - hepatitis B vaccine non-seroresponders; Fluvax – seasonal influenza vaccine. Mann Whitney test of medians. *p=0.0286

a



b



3.2.7 Induction of Tregs occurs in HBV non-responders in HD

Tregs were enumerated at baseline and day 7 after either seasonal influenza vaccine or HBV in healthy controls and CKD, and examined for expression of PD-1 and ICOS. Figure 27a provides an example of the gating strategies employed. There was no difference in baseline numbers of Tregs, defined as CD3⁺CD4⁺CD127^{lo}CD25^{hi}FOXP3⁺ cells, and expressed as a proportion of CD3⁺ cells, between HC and HD (*data not shown*). There was also no difference at baseline in numbers of Tregs expressing PD-1 or ICOS (*data not shown*). However, HBV non-responders demonstrated an increase in Tregs after HBV when compared to serological responders (fig. 27b)). However, there was no direct correlation with HBsAb titre in these patients. No significant difference was seen between HC and HD after seasonal influenza vaccine, and there was no correlation between change in Tregs and PB response in any group (*data not shown*).

Chapter 4: Discussion

End stage renal disease appears to be a state of immune deficiency,^{1,18,56} with increased risk of infection and of viral infection-related cancer.⁵⁷ Detailed information regarding the immune defects conferred by CKD, and whether these defects can be reversed by haemodialysis, has been difficult to obtain. This study characterized memory and effector T and B cell compartments in CKD and HD, and examined how these lymphocyte populations respond to vaccination.

Current vaccines depend for their efficacy on generation of high affinity antibodies, which depends in turn on affinity maturation within GC reactions, wherein proliferating B cells acquire somatic mutations in Ig genes, and are selected according to antigen affinity.^{35,58,59} Survival depends on provision of T cell help. TFH cells were first described as follicle-resident T cells, and exhibit a unique capacity to provide B cell help, including within GC.^{37,60,61} GC are relatively inaccessible in humans, but a subset of circulating memory T cells shares characteristics with GC TFH, including the capacity to provide B cells with help.^{43,44,61,62}

4.1 An intact CXCR3+ cTFH compartment is crucial for vaccine responses

The integrity of the CXCR3+ cTFH compartment, both numerically and functionally, appears to be crucial for adequate vaccine responses. At baseline, CKD patients are deficient in the CXCR3+ cTFH subset compared to healthy controls, and this is more pronounced in serological non-responders to HBV vaccination. By contrast, the CXCR3+ cTFH compartment appears to be numerically restored after regular haemodialysis, suggesting that lower proportions are a result of metabolic or

biochemical derangements due to CKD that can be corrected with haemodialysis. Despite this correction afforded by haemodialysis, baseline numbers of CXCR3+ cTFH does not predict subsequent serological response of HBsAb titre in these patients. Polymorphisms in CXCR5+, manifesting as reduced CXCR5+ expression and percentage of CXCR5+ cells within the CD4+ T cell compartment, are associated with an increased rate of serological non-response to HBV in healthy controls.⁶³ Interestingly, in the present study, there was no difference in proportions of CXCR5+ cells at baseline between CKD, HD patients and HC, including when analyzed according to HBV seroresponders and non-seroresponders. There are no described polymorphisms in CXCR3 which confer differences in vaccine responses.

After HBV, activation of the CXCR3+ cTFH compartment, as evidenced by upregulation of PD-1 expression, is defective in CKD patients when compared to healthy controls. PD-1 is normally upregulated after stimulation through the T cell receptor, and is a negative regulator of T cell activation and proliferation,⁶⁴ including on GC TFH where PD-1 is expressed at high levels.⁵⁸ In HC, the serological response to both seasonal influenza vaccine and HBV corresponds with induction of PD-1 expression by CXCR3+ cTFH. The defect in PD-1 induction in CKD is not absolute, since it was observed in CKD patients after seasonal influenza vaccine. This defect of activation appears to persist despite haemodialysis, and despite numerical restoration of the CXCR3+ cTFH compartment.

There was a strong correlation between induction of PD-1 on CXCR3+ cTFH and PB induction in healthy controls after seasonal influenza vaccine in 2012 and 2013.

This response was observed in both CCR7⁺ and CCR7⁻ subsets of cTFH, and although the response was of greater magnitude in the CCR7⁻ compartment, induction of PD-1 on CCR7⁺ cells correlated best with IgG PB response. These findings are consistent with the induction of PD-1 on cTFH as a readout of a specific and productive T-dependent B cell response within germinal centres. by contrast, the absence of a corresponding PB response after HBV indicates an origin of cTFH activation other than germinal centre(s), such as via activation of mature naïve T cells in a B cell-independent fashion³⁷. The defect in CKD would therefore appear to be within B-independent T cell responses.

ICOS is crucial to TFH development and function, including the formation of stable conjugates with antigen-specific B cells in germinal centres.^{55,65} ICOS expression correlates with PD-1 expression on cTFH in both health and autoimmune disease.⁴⁵ While upregulation of PD-1 reflects activation of cTFH after vaccination, by contrast, an increase in the number of ICOS⁺ expressing cells within the CXCR3⁺ cTFH compartment after vaccination is consistent with proliferation and release of these cells from germinal centres⁴². Proliferation of ICOS⁺ CXCR3⁺ cTFH cells after seasonal influenza vaccination is vaccine strain specific, and correlates with memory recall responses, but not naïve responses⁴². This study demonstrates that proliferation of cTFH in response to seasonal influenza vaccine is preserved in HD. Conversely, booster HBV does not elicit proliferation of cTFH, but based on this data it is not clear if this is confined to HD or would also be seen in healthy controls.

One possible model to account for these findings, and those reported previously, is that different cTFH subsets act at different stages in the generation of specific

antibody responses. Activation of CXCR3⁺ CCR6⁻ cTFH is observed in response to both novel and recall antigenic challenges, as suggested by their activation after both influenza vaccine and HBV. Conversely, CXCR3⁻ (predominantly CCR6⁺) cTFH correlate with the magnitude and maintenance of the subsequent antibody response, perhaps in response to repeated or chronic antigenic stimulus, as evidenced by their correlation with specific antibody responses in chronic autoimmune disease⁴³ and HIV⁴⁴. The integrity of serological immunity function depends on both baseline proportions of cTFH subsets and their activation state, as suggested by the apparent inability of hyper-activated, ICOS⁺ cTFH to support specific antibody responses in the elderly, and dysregulation of cTFH in autoimmune disease.

4.2 PB are a readout of boosted immunological memory

Overall, this study demonstrates that plasmablast responses to seasonal influenza vaccine are relatively preserved in patients with CKD or on HD, compared to healthy controls. This is consistent with serological data suggesting that whilst dialysis confers lower titres of vaccine-specific antibody production, there is no impairment of overall seroprotection rates.^{12,31} Seasonal influenza vaccines are thought to work mainly through boosting of immunological memory,^{23,66} with the presence of pre-immunization influenza-specific antibodies favours subsequent seroprotection. However, recent data are conflicting regarding whether pre-immunization titres contribute to impaired seroconversion in the context of HD.^{31,32} The present study did not examine serological responses to seasonal influenza vaccination, however plasmablast responses correlate closely with vaccine specific antibody after seasonal influenza vaccine^{38,39,48} and so can be used as a proxy

reading of specific antibody response in this context. The absence of a PB response after HBV, whether that is after the first dose of HBV in a naïve individual or after a booster dose in HD, suggests that this vaccine does not operate by boosting existing B cell memory when HBsAb are undetectable. This could be clarified by examining cellular responses to booster HBV in healthy controls.

The PB response to seasonal influenza vaccine seen in HC and CKD vaccinated in 2012/2013 was unable to be replicated in the 2015-2016 cohort. Whilst a PB response was seen in some individuals in this cohort, this was accounted for by an increase IgA PB, which account for most of the background circulating PB compartment³⁹, and are important in providing neutralizing antibody within the respiratory tract⁶⁶, suggesting these PB are the product of proliferating memory B cells. The seasonal influenza vaccines used for every year of both cohorts contained identical H1N1-like virus strain (A/California/7/2009). This strain was also used in the seasonal influenza vaccines administered in the Southern Hemisphere in 2011 and 2014⁶⁷. Interestingly, the “top” PB responder within the 2011-2013 cohort was the only individual in that cohort to have never previously received the seasonal influenza vaccine. Only two other individuals, both recipients of the 2015 seasonal influenza vaccine, had never previously received the seasonal influenza vaccine, and both demonstrated positive PB responses (albeit not the “top” responses) within a cohort which overall demonstrated no consistent PB response. One possible explanation is previous influenza infection in these individuals, which is superior in generating antigen-specific antibody compared with killed virus vaccines⁶⁶. Unfortunately this clinical information was not documented during the course of the study, and serum samples were not tested for pre-existing influenza specific antibodies. Alternatively, repeated

exposure to the same or similar antigens, through annual seasonal influenza vaccination, may have resulted in peripheral tolerance via induction Tregs. Furthermore, no change was seen in either percentages or activation markers of circulating Tregs, in either HC or HD. Other possible explanations include T cell anergy,⁶⁸ usually seen in states of persistent antigen, such as chronic viral infection, but which is unsubstantiated in the context of seasonal influenza vaccination; or direct interference from neutralizing pre-existing specific antibodies, which has been demonstrated in live intranasal, but not killed and intramuscularly administered, seasonal influenza vaccines.⁶⁶ Patients with CKD and those receiving HD are offered annual seasonal influenza vaccination as part of routine clinical care,^{11,69} and so by the time of recruitment in either cohort would have likely already received multiple annual vaccinations. The presence of pre-existing influenza-specific antibodies has been associated with impaired subsequent serological responses in HD in one study³¹, but this conflicts with other data³², and may simply represent repeated vaccinations in a high risk population. The healthy control groups in both cohorts were recruited mainly from health workers, who are also offered annual seasonal influenza vaccination as part of occupational medical care. It is possible, therefore, that the decline in PB response is a phenomenon witnessed in highly vaccination populations.

4.3 Regulatory T cells interfere with serological response to vaccination

This study has demonstrated, for the first time, that impaired serological response to HBV in patients receiving haemodialysis is attended by an increase in circulating Tregs. Infants with higher baseline frequencies of Tregs have impaired serological responses to measles vaccine, but do not demonstrate induction Tregs after

vaccination.⁵³ One possible explanation for this difference is that Tregs were enumerated at 4 weeks after measles vaccination, as compared to 7 days after vaccination in the current study. Alternatively, the difference may be attributable to vaccine-specific differences. Increased frequency of Tregs in peripheral blood has been reported after experimental autologous dendritic cell-based vaccination administered to individuals with HIV, but did not correlate with vaccine response.⁷⁰ Another recent study examined the effect of influenza vaccine on Tregs within tonsillar tonsils, which act as regulators of follicular helper T cell activity, called follicular regulatory T cells, or TFR, and found that vaccination results in a significantly reduced frequency of these cells in vaccinated children,⁷¹ suggesting that downregulation of regulatory factors enables expansion of influenza specific germinal centre TFH.

4.4 The effect of age on vaccine responses

The study population, including both CKD and HD patients, was predominantly elderly, which may have independently impacted the results. Another recent study identified a relative deficiency of cTFH in elderly individuals (without CKD),⁷² and also reported that cTFH cells in the elderly express higher levels of ICOS, consistent with a higher background level of T cell activation. The present study did not identify evidence for increased baseline cTFH activation in CKD or HD compared to healthy controls, measured by expression of either PD-1 or ICOS on cTFH and cTFH subsets. Similarly, there was no correlation between age and expression of PD-1+ or ICOS on cTFH in HC or any of the patient groups. Abundance of PD-1+ CXCR5+ cells has been shown to correlate with influenza vaccine responsiveness in the young but not in the elderly,⁷² which could account for why baseline PD-1 expression did not distinguish sero-responders, or between

CKD and HC, in a mostly elderly cohort. Similarly, other studies demonstrate an expansion of CXCR3⁺ cTFH subset in response to immediate antigenic challenge, particularly after influenza vaccination,⁴² but was not seen in this study of predominantly elderly HC and patients, suggesting a difference in this response between the elderly and children.

4.5 The augmentation of HBV dose is justified in CKD

Interestingly, a recent systematic review and meta-analysis of all available data⁸ regarding augmented dose HBV in CKD and patients receiving dialysis concluded there was insufficient evidence to support “double” dosing of these patients with 40µg of HBV. However, the present study has demonstrated for the first time that a four-dose schedule of 40µg of HBV is superior to a 20µg four-dose schedule when administered to patients with CKD prior to initiation of dialysis. The augmented schedule resulted in superior rate and magnitude of seroconversion. Only the 40µg schedule elicited HBsAb titres greater than 100mIU/ml, a preferential cutoff in CKD patients commencing dialysis, as it is associated with longer duration of sero-protection.⁵ These results suggest that a four-dose regimen of 40µg should be administered routinely to all CKD patients when in preparation for dialysis. Unfortunately, this study was insufficiently powered to support a 40µg augmented HBV dose schedule in patients already receiving dialysis, and was unable to demonstrate whether haemodialysis reversed the defect associated with impaired responses to HBV in CKD.

HBV using a recombinant antigen conjugated with a TLR9-agonist exhibited improved seroconversion in CKD.⁷³ While the cellular mechanism to account for this response remains to be determined, one hypothesis is activation of Th-1 CD4+ T cells.⁷³ The findings reported here provide an opportunity to test the cellular basis of this finding, in order to optimize vaccine responses in patients with CKD.

Chapter 5: Conclusions and clinical recommendations

This study has demonstrated several novel findings pertinent to the clinical care of patients with CKD and HD. Firstly, seasonal influenza vaccination is as effective, at a cellular level, in these patients as in HC, and therefore should be administered to all patients on an annual basis. Secondly, this study has determined several cellular mechanisms underpinning impaired immune responses in CKD, such as to the hepatitis B vaccine, including numerical and functional inadequacy of the CXCR3⁺ compartment, as evidenced by reduced frequencies of circulating CXCR3⁺ cTFH, and impaired upregulation of PD-1. Furthermore, while it appears the numerical defect in CXCR3⁺ cTFH can be overcome by haemodialysis, other functional defects persist, as evidenced by impaired induction of ICOS⁺ cTFH in HD, and increased frequencies of peripheral Tregs in serological non-responders. Further work is required to determine whether these latter defects are present in CKD prior to the initiation of HD, or in HC who are poor sero-responders. And finally, an augmented dose schedule of HBV is justified in CKD patients in preparation for haemodialysis, as this results in improved rates and magnitude of seroconversion, approximating that seen in healthy controls who had been administered standard schedule HBV.

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